ARE MARKERS OF FIBRIN TURNOVER IN BLOOD AND URINE INCREASED BEFORE AND DURING EXACERBATION OF ASTHMA?

By

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This thesis is submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Medicine from the University of Portsmouth

ABSTRACT

INTRODUCTION: Asthma is a chronic inflammatory disorder of the airways punctuated by acute exacerbations. Coagulation and fibrinolysis pathways are increasingly recognised in the asthmatic inflammatory milieu and markers of fibrin turnover represent potential asthma biomarkers targets. A biomarker in plasma or urine predicting exacerbation could enable early intervention to attenuate or prevent exacerbation.

STUDY DESIGN: A study in 2 parts comparing exacerbation and stable asthma was performed. Part 1 compared adults with exacerbations requiring hospital treatment to clinical recovery. Part 2 prospectively followed a cohort of adult moderate to severe asthmatics when well until exacerbation and through to clinical recovery.

METHODS: Plasma was analysed by ELISA for markers of fibrin turnover alongside inflammatory and cellular markers, and comparison was made between exacerbation and recovery. Urine was analysed by latex agglutination for fibrin(ogen) degradation products (FDPs). Symptoms were recorded and a score was derived to distinguish asthma exacerbation from baseline and recovery states. Comparison was made between exacerbation and recovery using multivariate statistical analysis.

KEY FINDINGS: Prospectively, the detection of FDP in urine significantly increased the likelihood of subsequent asthma exacerbation in the following 7-14 days between 4 and 6 times that of a negative urine FDP test. FDP +7 days (OR 4.35 (95% CI 1.12, 16.9) p=0.03), FDP +10 days (OR 5.50 (95% CI 1.38, 21.9) p=0.02), FDP +14 days (OR 6.07 (95% CI 1.43, 25.8) p=0.02). Plasma D-dimer was significantly increased from baseline to exacerbation (median 0.07 µgFEU/ml (95% CI -0.01, 0.15) p=0.02). Plasma eosinophils (median $10^{*}9$/l (95% CI 0.10, 0.29) p=0.02) and eotaxin (median 12 pg/ml (95% CI 3, 22) p<0.001) increased significantly from exacerbation to recovery, and PF4 significantly increased from exacerbation to follow up (median 9 µg/ml (95 % CI 2, 17), p=0.002). Positive correlations were observed at exacerbation between eosinophils and eotaxin (p<0.001), platelets and PF4 (p=0.02) and platelets and eosinophils (p=0.01). Clinical symptoms and reliever

use increased in the 10 days preceding exacerbation and reduced during recovery ($p<0.001$).

CONCLUSIONS: This is the largest study to date examining coagulation and fibrinolysis in acute exacerbation of asthma. Detection of urine FDP in moderate to severe asthma suggests up to 6-fold increased exacerbation risk in the subsequent 14 days. The potential to translate these findings into a point-of-care test for use by patients at home warrants further study. Positive correlations between eosinophils, eotaxin, platelets and platelet factor 4 (PF4) alongside increased PF4, eosinophils and eotaxin in plasma after a period of recovery and treatment support eosinophil and platelet migration into the lung during asthma exacerbation. The symptom score used in this study provides a simple objective measure of asthma exacerbation for use in future research or clinical practice but requires prospective validation.

Declaration:

"Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award."

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LIST OF ABBREVIATIONS

AHR	Airway Hyperresponsiveness
ALI	Acute lung injury
ANOVA	Analysis of variance
APC	Activated protein C
APTR	Activated partial thromboplastin ratio
APTT	Activated partial thromboplastin time
ARDS	Acute respiratory distress syndrome
ARTP	Association for respiratory technology and physiology
AT	Antithrombin
ATS	American thoracic Society
BALF	Bronchoalveolar lavage fluid
BDP	Beclometasone dipropionate
BMI	Body mass index
BNHFT	Basingstoke and North Hampshire NHS Foundation Trust
BSA	Bovine serum albumin
BTS	British Thoracic Society
BU	Biological unit
CLRN	Comprehensive local research network
COPD	Chronic obstructive pulmonary disease
CRF	Case record form
CRP	C-reactive protein
CXR	Chest x-ray
ECCS	European Community of Coal and Steel
ECM	Extracellular matrix
ECP	Eosinophilic cationic protein
ED	Emergency Department
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
EMT	Epithelial-mesenchymal transition
EMTU	Epithelial mesenchymal trophic unit
ERS	European Respiratory Society
FBC	Full blood count
FCS	Fetal calf serum

FDP	Fibrin(ogen) degradation product
FeNO	Fractional exhaled nitric oxide
FEU	Fibrinogen equivalence unit
FEV_1	Forced expiratory volume in 1 second
FGF	Fibroblast growth factor
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
GAG	Glycosaminoglycan
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte macrophage colony stimulating factor
HK	High molecular weight kininogens
HRP	Horseradish peroxidase
ICS	Inhaled corticosteroids
IFN	Interferon
IgE	Immunoglobulin E
IGF	Insulin-like growth factor
IL	interleukin
INR	International normalised ratio
IPF	Idiopathic pulmonary fibrosis
IT	Information technology
LABA	Long-acting $beta_2$ agonist
LFT	Liver function test
LTRA	Leukotriene receptor antagonist
MAU	Medical Assessment Unit
MMP	Matrix metalloproteinase
NAC	N-acetyl cysteine
NHBE	Normal human bronchial epithelial cells
NHLBI	National Heart Lung and Blood Institute
NHS	National Health Service
NIHR	National institute for health research
NO	Nitric oxide
$PaCO_2$	Partial pressure of arterial carbon dioxide
PAI	Plasminogen activator inhibitor
PAF	Platelet activating factor

PAR	Protease activated receptor
PBS	Phosphate buffered saline
PCRN	Primary care research network
PDGF	Platelet-derived growth factor
PEFR	Peak expiratory flow rate
PF4	Platelet factor 4
PFT	Pulmonary function test
PHT	Portsmouth Hospitals NHS Trust
PI	Principal Investigator
PIC	Participant identification centre
PIS	Participant information sheet
PK	Prekallikrein
Plats	Platelets
PMHx	Past medical history
PT	Prothrombin time
PTFE	Polytetrafluoroethylene
QAH	Queen Alexandra Hospital
RANTES	Regulated on activation normal T-cell expressed
REC	Research ethics committee
SABA	Short-acting $beta_2$ agonist
SAE	Serious adverse event
SBU	Standardised biological unit
serpin	Serine protease inhibitor
SIGN	Scottish Intercollegiate Guideline Network
SMART	Symbicort as maintenance and reliever therapy
SPT	Skin prick testing
SR	Slow release
TAFI	Thrombin-activatable fibrinolysis inhibitor
TB	Tuberculosis
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
tPA	Tissue-type plasminogen activator
TGF-β	Transforming growth factor beta
Th	T helper lymphocyte

TM	Thrombomodulin
TMB	Tetramethylbenzidine
TNF	Transforming growth factor
UoP	University of Portsmouth
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
U+E	Urea and electrolytes
VEGF	Vascular endothelial growth factor
Xase	Ten-ase

DEDICATION

To my wife Jo for her ceaseless support and patience, I couldn't have done this without you.

To Lucy and Charlotte, thank you for the distractions. Can we go and play now?

1.0 INTRODUCTION

1.1 Asthma

1.1.1 Definition

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: in particular, mast cells, eosinophils, neutrophils, T lymphocytes, macrophages, epithelial cells and airway smooth muscle. In susceptible individuals, this inflammation causes recurrent episodes of coughing (particularly at night or early in the morning), wheezing, breathlessness, and chest tightness. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment (1).

Although often thought of as one disease, asthma is really a spectrum of clinical and inflammatory phenotypes that affect individual asthma sufferers in dramatically different ways. The severity of asthma varies greatly from mild disease that causes little in the way of symptoms and can be well controlled with minimal intervention, to severe disease causing daily debilitating symptoms and frequent life threatening exacerbations despite extensive therapy and polypharmacy.

1.1.2 The burden of asthma

300 million people worldwide suffer from asthma and its prevalence increases by 50% every decade (2). In the UK, 5.1 million people have asthma and over half (2.6 million) suffer serious symptoms, including debilitating breathlessness, attacks so bad they cannot speak, fear that they may die and emergency hospital admissions (3). Worldwide, 180,000 deaths each year are attributable to asthma (2). In 2004 the British Thoracic Society (BTS) reported 1381 deaths from asthma across the whole of the UK (4).

The financial burden of asthma is enormous and it is estimated that developed countries will spend between 1 and 2% of their healthcare budget treating the condition (2). Not surprisingly, more severe disease consumes a larger proportion of the expenditure than mild disease and 80% of asthma expenditure goes on the 20% of sufferers with more severe disease. In the UK over £45.8 million are spent on emergency hospital admissions for asthma and for every 2 people admitted to hospital, 5 more are treated in the Emergency Department. Asthma that is difficult to control costs the National Health Service (NHS) £680 million every year, and results in 18 million lost working days to the UK economy (3).

1.1.3 Asthma exacerbation

The term asthma exacerbation is commonly used, yet poorly defined. International guidelines refer to a progressive increase in shortness of breath, cough, wheezing, or chest tightness, or some combination of these symptoms. They are further characterised by decreases in expiratory airflow measured by spirometry or peak expiratory flow rate (PEFR) (1, 5). The Global Initiative for Asthma (GINA) guideline (5) also refers to a requirement for symptoms sufficient to require a change in treatment. BTS (4), GINA (5) and National Heart Lung and Blood Institute (NHLBI) (1) guidelines all give definitions of exacerbation severity based on physiological parameters such as PEFR, heart rate, oxygen saturation, arterial partial pressure of carbon dioxide ($PaCO_2$), in combination with clinical parameters such as the ability to complete a sentence, auscultatory findings and clinical exhaustion. However, these definitions are applicable to an established exacerbation, which we can see from the above definition is somewhat nebulous.

Historically from a research perspective, the definition of an exacerbation has varied greatly from one study to the next. The American Thoracic Society (ATS) and European Respiratory Society (ERS) recently jointly set up a task force to agree definitions for different aspects of asthma which included exacerbation (6). They define a severe exacerbation as events that require urgent action on the part of the

patient and physician to prevent a serious outcome such as hospitalisation or death from asthma. Furthermore, a severe exacerbation should include at least 3 days of oral corticosteroid use.

For an asthma patient, an exacerbation may range from a mild increase in symptoms, to a life threatening episode of breathlessness. Unsurprisingly, exacerbations are one of the most frightening and dangerous aspects of asthma and sufferers fear for their lives. In a recent publication by Asthma UK, patients describe asthma as "living with a time bomb", and exacerbations as "like I'm going to die". One father explained "I simply had no idea asthma could be that serious, not until the day my daughter died in my arms" (3).

Taking both clinical guidelines and research definitions into account, whether an asthma exacerbation has occurred remains subjective on the part of both patient with respect to symptoms, and clinician with respect to whether treatment is warranted. Although quantifiable parameters such as PEFR or forced expiratory volume in 1 second (FEV_1) are more objective, in the absence of symptoms, worsening airflow alone does not mean an exacerbation is occurring. The use of percentage change in PEFR from baseline as a marker of exacerbation has been comprehensively reviewed by the ATS/ERS task force and is not currently recommended as a marker of severe exacerbation (6).

1.1.4 Recovery from exacerbation

Although attempts have been made to define an exacerbation, neither clinical nor research guidelines define subsequent recovery from an exacerbation. As recognised by the ATS/ERS task force, exacerbations are identified as events characterised by a change from the patient's previous status, a concept that should be applied in clinical trials (6). BTS guidance describes the key event in recovery with respect to criteria and timing for hospital discharge, as being improved symptoms and PEFR rather than a complete return to normality (4).

Although there is a paucity of guidance in this area, the inference from these statements is that recovery from exacerbation is a return to the patient's previous status of "normality". This does not take into account previous asthma control, which may be poor, rather recovery is a return to the patient's pre-exacerbation state.

1.1.5 Asthma control

From a clinical perspective it is useful to think of asthma control and exacerbation as separate entities, accepting that poor asthma control is likely to result in more frequent exacerbation.

Good asthma control may require significant medications to maintain, and may not be achieved in every case. GINA determines asthma control using the following criteria (5):

- Asthma symptoms
- Limitations of activities
- Nocturnal symptoms/awakening
- Need for reliever/rescue medication
- Exacerbations
- Lung function (PEFR or FEV_1)

GINA defines controlled asthma as having normal lung function with none of the other criteria above.

1.1.6 Classification of asthma

The classification of asthma has been the subject of much debate in the past. A variety of terms are in use and although they help to define certain disease characteristics, they are not always helpful in disease management. "Atopic asthma" for example, may be useful to describe an individual who demonstrates sensitisation to common aeroallergens, however allergen avoidance may have no impact on their disease and may not be practical. Asthma is often termed "difficult" or "difficult-to-control" but this definition has no agreed parameters.

With regard to asthma severity, the ATS/ERS task force (6) agreed to classify asthma in terms of the difficulty of controlling symptoms, i.e. how much treatment is required, or not controlled despite significant treatment. Although not explicitly stated in the past, this has been implied for a number of years from treatment guidance in American and British asthma guidelines. GINA guidance now adopts a similar approach but their previous classification based on symptoms prior to treatment is still recognised as having a potential role for research purposes (7).

The BTS and Scottish Intercollegiate Guidelines Network (SIGN) asthma guidelines (4) have, for a number of years, recommended a stepwise approach to therapy [Figure 1], adding or subtracting medication to achieve asthma control.

A similar approach is recognised by the latest GINA and NHLBI guidelines (1, 5), albeit with minor differences in inhaled steroid dose brackets and the order in which drugs are stepped in. A further criteria was published in 2000 by the ATS defining severe refractory asthma by a combination of treatment required to achieve control and criteria similar to those used by the 2008 GINA guidelines, however this criteria is not commonly used in clinical practice (8).

Figure 1 - BTS stepwise management of asthma in adults

Adapted from BTS asthma guideline 2011 – summary of stepwise management in adults	
Step 1 Mild intermittent asthma	Inhaled short-acting beta$_2$ agonist (SABA) as required
Step 2 Regular preventer therapy	Add inhaled corticosteroid (ICS) 200-800mcg/day • 400mcg/day is an appropriate starting dose for many patients • Start at dose of ICS appropriate to severity of disease
Step 3 Initial add-on therapy	1. Add inhaled long-acting beta$_2$ agonist (LABA) 2. Assess control of asthma: • Good response to LABA – continue LABA • Benefit from LABA but control still inadequate – continue LABA and increase ICS to 800mcg/day • No response to LABA – stop LABA and increase ICS to 800mcg/day. If control still inadequate, institute leukotriene receptor antagonist (LTRA) or slow-release (SR) theophylline
Step 4 Persistent poor control	Consider trials of: • Increasing ICS up to 2000mcg/day • Addition of a 4th agent – e.g. LTRA, SR theophylline, β_2 agonist tablet
Step 5 Continuous or frequent use of oral steroids	Use daily steroid tablet: • In lowest dose providing adequate control • Maintain high dose ICS at 2000mcg/day • Consider other treatments to minimise the use of steroid tablets • Refer patient to specialist care
Note all doses refer to BDP (beclomethasone dipropionate) or equivalent – see appendix **1**	

1.1.7 Brittle asthma

There is a subset of patients who are said to have “brittle” asthma due to the rapidity of deterioration when exacerbation occurs. Type I brittle asthma is determined by persistent diurnal variation of airflow limitation of >40% for >50% of the time over a period of 150 days despite multiple drug therapies, i.e. poor control measured by airway hyperresponsiveness despite treatment. Patients with type II brittle asthma are usually well controlled but once exacerbation occurs, deteriorate rapidly and very severely (9). It is important to highlight that while such subtypes are described, they rarely occur as an isolated clinical phenomenon and do not always align with recognised inflammatory phenotypes.

1.2 Asthma pathophysiology

Here is described the pathology and key inflammatory events in asthma, in order to discuss how they may be implicated in the coagulation cascade, which forms the basis of the hypothesis tested in this thesis.

1.2.1 Asthma development and onset

Asthma is long established as an inflammatory disorder, particularly characterised by a type II T helper lymphocyte (Th2) inflammatory profile. However, not all atopic individuals develop asthma and asthma occurs in the absence of atopy. Other factors must therefore be present for asthma to occur. A family history of asthma is recognised as a risk for disease development (10) however, numerous candidates exist for asthma susceptibility genes (e.g. ADAM 33) and their roles and complex interactions remain incompletely understood (11). The observation that basement membrane thickening is present in the airways of asthmatic children in the absence of prolonged inflammation (12) has opened the possibility that existing structural alterations may precede and even be a prerequisite for the inflammatory response. This model proposes the “soil” of structural abnormality enabling the “seed” of airway injury to result in chronic inflammation and subsequent remodelling (13). Basement membrane thickening occurs in children in the absence of inflammation

and before clinical onset of asthma. Furthermore, this airway remodelling does not occur with childhood wheeze alone (14).

1.2.2 Epithelial fragility

The bronchial epithelium forms a protective barrier against inhaled agents. In asthma the epithelium demonstrates increased fragility (15), possibly by reduced desmosomal contact in this group (16, 17). Once columnar epithelium is denuded, basal cells rapidly flatten and spread to cover the basement membrane, accompanied by plasma exudation that results in a gel-like network of fibrin and plasma as well an inflammatory cell infiltration (18). The normal epithelial barrier is rapidly reformed by migration and proliferation of existing epithelial cells which then undergo apoptosis to restore cell numbers and proportion of cell types (19).

1.2.3 Epithelial injury

There is an exaggerated epithelial inflammatory response to insult in asthma resulting from a range of stimuli including allergens and viruses. Once sensitised to an allergen, the production of specific immunoglobulin E (IgE) enhances the inflammatory response to subsequent exposure through mast cell degranulation (20). In the case of viral pathogens, asthmatic airway epithelium not only produces inflammatory cytokines but undergoes apoptosis as a defence mechanism to reduce virus replication in infected cells (21). Recurrent epithelial damage resulting from these multiple hits induces a state of chronic inflammation in the asthmatic airway (22).

1.2.4 Airway remodelling

As described above, structural airway abnormalities in the form of thickened basement membrane occur in asthma but not wheezy bronchitis in children (14). Recurrent inflammatory insults and the chronic inflammatory state that ensues, result in a process of airway remodelling. This consists of thickening of the basement membrane, smooth muscle hypertrophy and hyperplasia, and vascular proliferation. However, this is not explained entirely by inflammation as

demonstrated by progressive airway remodelling in more severe asthma despite high-dose corticosteroids. In fact, airway remodelling may be a protective measure as individuals with more extensive remodelling, display less bronchial hyperreactivity (13).

The epithelial mesenchymal trophic unit (EMTU) is a term used to describe the interaction of the epithelium with underlying mesenchymal tissue. This communication is observed in embryology during lung development (23), it is proposed that reactivation of the EMTU occurs in asthma and mediates airway remodelling (14). Injury to the asthmatic epithelium results in release of pro-fibrogenic growth factors including fibroblast growth factor (FGF)-2, insulin-like growth factor (IGF)-1, platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β) 2 (13). In response to profibrogenic stimuli, subepithelial fibroblasts undergo transformation to a myofibroblast phenotype with resulting alterations in extracellular matrix deposition and airway remodelling (24).

1.2.5 Inflammatory phenotypes

Increasingly, there is recognition that the heterogeneity of asthma is, in part, due to differences in inflammatory phenotype. The use of bronchoalveolar lavage, bronchial biopsy and sputum induction enables examination of the cellular inflammation within the airway lumen. Four cellular phenotypes are now recognised; eosinophilic, neutrophilic, paucicellular and mixed (25). Sputum induction has certain advantages over bronchoscopic evaluation in that it is less invasive, requires less personnel and equipment, and repeat examination can be performed with relative ease enabling comparison after intervention.

Looking solely at airway luminal inflammation has its limitations e.g. mast cells in asthma are usually located in the airway smooth muscle, mucous glands and epithelium (26) but not readily found in the airway lumen. Nonetheless, relationships between inflammatory and clinical phenotypes are recognised and useful in practice (27).

1.2.6 Eosinophils

Eosinophils derive from CD34+ bone marrow precursors in response to Th2 cytokines including GM-CSF (granulocyte macrophage colony stimulating factor), interleukin (IL)-3 and IL-5. IL-5 and eotaxin have chemoattractant effects on eosinophils and IL-4 and IL-13 generated in target organs increase eosinophil adhesiveness (28). Eosinophils have numerous roles including antigen presentation, regulation of T cell function and granulocytes, and storage of numerous preformed cytokines (29, 30). Eosinophils are also a source of TGF-β and may play a role in airway remodelling (31).

Much of the recent focus on eosinophils stems from studies demonstrating successful outcomes from intervention to reduce eosinophilia. Green and colleagues (32) studied the use of sputum eosinophilia to guide corticosteroid therapy compared with standard guideline treatment. They demonstrated that by increasing steroids and reducing eosinophilia there was a reduction in asthma exacerbations and hospital admissions compared with standard treatment. Moreover, no adverse outcomes were observed when steroids were reduced in the absence of eosinophilia.

Steroids, however, affect not only eosinophils and initial studies of the anti-IL-5 monoclonal antibody mepolizumab showed a lack of significant clinical outcomes despite reducing eosinophils (33, 34). More recent studies have demonstrated positive outcomes using mepolizumab. In subjects with persistent eosinophilia despite corticosteroids, eosinophils were significantly reduced, as were exacerbation rates (35, 36). These observations demonstrate that the eosinophil is an important player in the inflammatory milieu, but to be effective, treatments must also affect other aspects of the inflammatory process.

1.2.7 Neutrophils

The role of the neutrophil in asthma is less clear. A neutrophil predominance in asthmatic airways is associated with steroid resistance (37), and is observed in

acute and chronic infection, smoking, obesity and with exposure to occupational antigens, endotoxin exposure and environmental pollutants (38). There is some evidence that macrolides may be of benefit in this group via an anti-inflammatory mechanism that is independent of their antibiotic effect (39).

1.2.8 Inflammatory cytokines

The balance of inflammatory and inhibitory cytokines determines the cell types present in the airways and therefore certain characteristics of an individual's disease. Th2 cytokine predominance promotes IgE production (IL-4), eosinophil maturation and survival (IL-5) and airway hyper-responsiveness (AHR) (IL-13) (40). Viral infection is thought to be the trigger for asthma exacerbation in around 70% of cases (41) and is associated with increased IL-8 (a neutrophil chemoattractant), RANTES (regulation on activation normal T-cell expressed) (a T-cell chemoattractant) and pro-inflammatory cytokines such as TNFα (tumour necrosis factor) (21, 42). Inhibitory cytokines such as IL-12 suppress Th2 responses and may shift the balance in favour of the Th1/neutrophilic inflammation associated with steroid-resistant refractory asthma (43). There is also emerging evidence to support a role for Th17 cells and their cytokines including IL-17a in severe steroid-resistant asthma, a T cell bias mediated via IL-6 (40).

1.2.9 Immunoglobulin E (IgE)

IgE mediates allergic reactions and sensitisation to allergens with the development of specific IgE to aeroallergens such as pollen, hastens this effect. IgE attaches to cell surfaces via high-affinity receptors on mast cells, basophils, dendritic cells and lymphocytes. Exposure to specific allergen induces crosslinking of IgE on the cell surface and degranulation results in the release of inflammatory cytokines that perpetuate inflammation and induce bronchospasm (44). Targeted therapy with monoclonal antibodies against IgE (omalizumab) has been shown to be effective in reducing exacerbations and corticosteroid requirements (45).

1.2.10 Exhaled nitric oxide

Nitric oxide (NO) has vasodilator, bronchodilator, neurotransmitter and inflammatory mediator roles and is detectable at high levels in exhaled breath in asthma (46). The fractional exhaled nitric oxide (FeNO) is considered as a surrogate marker of eosinophilic airway inflammation (47). Although studies examining clinical outcomes based on treatment in response to serial FeNO measurements have so far been disappointing (48), it is considered a useful and non-invasive measure in the management of asthma. Recent ATS guidance on the use of FeNO recommends it is used in the diagnosis of eosinophilic airway inflammation, determination of steroid-responsiveness and in support of an asthma diagnosis where objective evidence is required (49). The National Institute of Health and Care Excellence have also recently approved it for diagnosis and treatment of asthma (50).

1.2.11 Mechanical stimulation

Epithelial damage results not only from inflammatory insult but also mechanical stimulation as occurs during bronchospasm. Communication between the epithelium and extra cellular matrix (ECM) occurs under such circumstances as demonstrated in experimental models (51, 52), and impaired wound repair has also been demonstrated (53). Recent biopsy studies have demonstrated that this also occurs *in vivo* in the absence of an inflammatory stimulus to bronchoconstriction (54).

1.2.12 Plasma exudation

The airway mucosa encompasses an extensive microvascular network which is highly responsive to inflammatory vasopermeability agents including histamine, bradykinins, leukotrienes, platelet-activating factor (PAF), eosinophilic cationic protein (ECP), tryptase and TNFα (55). In response to such stimuli, gaps appear between endothelial cells of venules allowing plasma exudation into the lamina propria (56). Under normal conditions, a minor baseline transfer of plasma occurs that selectively favours smaller proteins, however, under exudative conditions

plasma is transferred in bulk with no filtration of plasma. Furthermore, the bulk plasma proceeds across the epithelium into the airway lumen without significant filtration. In plasma, the ratio of albumin (60kDa) to α_2-macroglobulin (700kDA) is 20:1, in resting nasal lavage this ratio is 40:1 indicating retention of larger proteins within the plasma. Under exudative conditions the albumin (60kDa) to α_2-macroglobulin (700kDA) ratio in nasal lavage is equal to that of plasma (20:1) demonstrating the passage of unfiltered plasma into the lamina propria and across the respiratory epithelium (57).

It is important to highlight that although plasma exudation occurs in response to inflammatory stimuli, the passage of bulk plasma into the airway lumen occurs across an intact epithelium. Plasma proteins pass between the epithelial cell-cell junctions onto the luminal surface in response to elevated pressure in the lamina propria and the resulting transepithelial pressure gradient (58). This transepithelial passage is unidirectional and mucosal permeability is not affected under these conditions (57).

This exudation of plasma into the airway lumen is thought to transport cytokines, immunoglobulins and plasma proteins, including coagulation factors.

1.2.13 Matrix metalloproteinases

The matrix metalloproteinase (MMP) family are a group of enzymes involved in extracellular matrix remodelling, cellular migration and are implicated in lung epithelial repair mechanisms (59). In the asthmatic airway, MMPs are thought to be involved in airway remodelling but may have differing roles. MMP-2 and MMP-3 have been implicated as promoters of airway remodelling (60), whereas MMP-9 may have a protective effect (61). Suppression of MMP-9 activity by plasminogen activator inhibitor-1 (PAI-1) is associated with increased ECM deposition (62).

1.2.14 Glycosaminoglycans

The glycosaminoglycans (GAGs) are a family of carbohydrates that include anticoagulants such as heparin and the naturally-occurring heparan sulphate, and extracellular matrix components such as hyaluronan. Fibroblasts in the asthmatic airway produce increased hyaluronan, implicating this protein in asthmatic airway remodelling (63). Heparin, usually considered an anticoagulant is known to possess anti-inflammatory properties, possibly mediated via the coagulation cascade. Furthermore, it is highly sulphated and therefore highly negatively charged allowing it to bind to a wide range of biological materials. These include TGF-β, ECP, cytokines and chemokines, and heparin binding can inhibit many of these factors (64). Endogenous heparin levels are low in asthma implying these anti-inflammatory properties are absent in asthma (65). However, in a murine model of airway inflammation, defective production of heparan sulphate resulted in reduced airway inflammation, possibly via reduced eosinophil migration, implying a dual role for these proteins (66).

1.2.15 Transforming growth factor beta-1 and vascular endothelial growth factor

Transforming growth factor beta-1 (TGF-β1) is one isoform of the TGF-β superfamily of profibrotic cytokines. It is expressed by bronchial epithelium in the lung and from the majority of inflammatory cells involved in the asthmatic response including eosinophils (67). TGF-β1 promotes airway remodelling in asthma, probably via epithelial mesenchymal transition (EMT), an embryonic developmental process reactivated in wound healing and repair (68, 69). The observation that there is a positive correlation with plasma levels of TGF-β1 and uncontrolled asthma suggests it may be a marker of asthma control (70). Furthermore, TGF-β1 stimulates lung fibroblasts to produce vascular endothelial growth factor (VEGF) (71).

VEGF has effects on angiogenesis, vascular permeability, leukocyte migration and proliferation of smooth muscle and endothelial cells (72). With this in mind, it is perhaps unsurprising that elevated sputum (73), serum and plasma levels of VEGF

are observed in stable asthma when compared with healthy controls, and that these levels rise during exacerbation (74).

1.3 Therapeutic goals

It is clear that reducing asthma symptoms and exacerbations would be beneficial to patients by improving quality of life and reducing morbidity and mortality. Furthermore, there are significant societal gains to be made in terms of direct and indirect financial cost. The translation of cellular and inflammatory phenotype into personalised titration of corticosteroids or, monoclonal antibody therapy, is a significant step towards achieving these gains from the historical perspective of blind steroid prescribing supported by beta agonists. There are, however, still a significant number of individuals who continue to suffer unpredictable and life-threatening exacerbations despite these interventions.

By definition asthma symptoms and airflow limitation are variable and there must be sufficient increase in symptoms or decrease in markers such as PEFR for patients to seek medical help or commence treatment as per self-management plans. Tattersfield and colleagues (75) investigated acute asthma exacerbation and observed that five days prior to commencing treatment for exacerbation when symptoms peak and PEFR levels trough, patients experience a gradual increase in symptoms accompanied by a gradual decline in PEFR which accelerates over these five days. This phenomenon has been utilised by treatment strategies such as Symbicort SMART (symbicort maintenance and reliever therapy) whereby the use of ICS combined with a LABA as preventer and reliever can reduce the frequency and severity of exacerbation (76, 77). However, for those with more severe and brittle disease, once the symptoms of an exacerbation have begun it is too late to prevent an exacerbation.

The detection of inflammatory changes occurring pre-exacerbation could enable intervention in even the most severe asthmatic with the goal of moderating or even preventing exacerbation. Existing inflammatory indices such as sputum eosinophilia

are not practical for the frequent, possibly daily monitoring that would be required, whereas modification of a marker such as FeNO that might be practical has not been shown to affect exacerbations.

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention. In clinical terms this is a laboratory measurement or physical sign used as a surrogate marker of clinical meaningful outcome (78). A successful biomarker requires sufficient sensitivity, specificity, positive predictive value and negative predictive value to identify a predetermined clinical state in the absence of established diagnostic criteria with sufficient accuracy to delineate presence and absence of that clinical state (79). Applying this to asthma exacerbation, an effective biomarker would need to predict an exacerbation of asthma prior to development of clinical signs or symptoms with sufficient accuracy to justify intervention and its inherent complications balanced against the risks of exacerbation occurring.

A suitable biomarker of asthma exacerbation that is safe, easily measured, cost effective and enables successful intervention and outcomes is yet to be found. Testing such a biomarker would need to be possible by patients themselves, similar to a capillary blood glucose monitor for diabetes, or a urinary pregnancy test. In line with the changes in lung function observed by Tattersfield and colleagues 5 days before an exacerbation (75), Brims et al observed a dramatic increase in coagulation markers in the sputum of an asthmatic displaying no signs or symptoms, who five days later suffered an exacerbation of his asthma (80). The coagulation cascade is therefore a promising target for such a biomarker and is discussed in detail below.

1.4 Haemostasis

In order to understand the roles of fibrinogenesis and fibrinolysis in the lung one must first understand coagulation in the more familiar setting of haemostasis.

Haemostasis describes several processes which occur in response to endothelial injury resulting in the formation of a clot that temporarily plugs the area of damage, allowing repair and restoration of the endothelium. Once the endothelial barrier is restored, the clot is removed by fibrinolysis restoring vessel patency and integrity. In reality haemostatic and fibrinolytic mechanisms occur simultaneously and it is the balance of clotting factors that determines which is the dominant process at any particular time. For the purposes of understanding it is useful to separate each process before looking at the complex interactions that continuously occur between them.

1.4.1 Platelets

The first response to endothelial damage is platelet adhesion to exposed extracellular matrix at the site of injury, followed by platelet aggregation and subsequent activation. Activated platelets release the contents of cytoplasmic alpha granules which include coagulation factors, cytokines, chemokines, growth factors involved in the inflammatory response and platelet factor 4 (PF4) (81). Furthermore, the platelet surface acts as a platform for certain coagulation factors to bind in a configuration that optimises their enzymatic action (82) as described below.

1.4.2 Coagulation

The process of coagulation, or fibrinogenesis, is an enzymatic cascade that results in the generation of a stable cross-linked fibrin clot. It encompasses the intrinsic and extrinsic cascades and final common pathway. The term extrinsic arises because tissue factor (TF), the initiator of this pathway is not expressed on cells exposed to circulating blood, whereas the intrinsic cascade consists entirely of factors within

the circulation. This division is far less straightforward in reality as will be seen, but is nevertheless useful in describing and understanding fibrinogenesis (see Figure 2).

1.4.3 The extrinsic pathway

TF is not present on vascular endothelium, but is widely expressed on extravascular cell surfaces. TF is expressed in a pattern that envelopes the vascular tree, however, the density of expression is variable and represents a response to the differential importance of bleeding in different anatomical sites (83). Endothelial damage exposes extravascular TF which binds with circulating activated factor VII (FVIIa). FVII is activated by proteolytic cleavage by TF dependence or downstream serine protease inhibitors (serpins) including FXa, FIXa, FXIa, FXIIa, thrombin and plasmin (84). The TF-FVIIa complex cleaves factor X (FX) and is known as the extrinsic "tenase" (Xase). The resulting FXa is the beginning of the common pathway and in turn activates prothrombin by further enzymatic cleavage to form thrombin.

1.4.4 The intrinsic pathway

The intrinsic pathway begins with factor XII (FXII) which undergoes autoactivation when exposed to the anionic surface of ECM. FXIIa formation leads to prekallikrein (PK) activation in the presence of high molecular weight kininogens (HK) and it is this interaction that gives rise to the pseudonym "contact pathway" (85). Importantly this provides an alternative activator of coagulation and hence complimentary mechanisms of action. FXIIa in turn cleaves FXI, FXIa in the presence of Ca^{2+} activates FIX which combines with its co-factor FVIIIa to form the FIXa-FVIIIa complex known as the intrinsic Xase, which in turn activates FX to FXa beginning the final common pathway (86).

1.4.5 The final common pathway

Both the extrinsic and intrinsic pathways result in the production of FXa, then follows the final common pathway. FXa binds with its co-factor factor Va and the

resultant FXa-FVa complex, also known as the pro-thrombinase complex, more effectively cleaves prothrombin to thrombin. Following this model, thrombin now cleaves fibrinogen forming fibrin monomers which are then crosslinked by activated factor XIII (FXIIIa) producing the final product, a stable polymerised fibrin clot (86).

1.4.6 Fibrinogenesis *in vivo*

This division into extrinsic, intrinsic and common is useful for understanding coagulation *in vitro* and is the basis of the laboratory tests for Prothrombin Time (PT; tests extrinsic and common) and Activated Partial Thromboplastin Time (APTT; tests intrinsic and common). However, *in vivo*, the extrinsic Xase is only capable of producing a small amount of thrombin which is not sufficient to cleave enough fibrinogen to form a fibrin clot. This thrombin acts as a primer which in turn can directly activate FXI without the need for FXII (87). The primer also activates FV and FVIII, without which the intrinsic pathway could not progress, and FXIII which completes fibrinogenesis by crosslinking fibrin monomers (86). It is the intrinsic pathway that produces thrombin in sufficient quantity to cleave enough fibrinogen to form a fibrin clot. To do this, it utilises the phospholipid surface of platelets as a platform to assemble and align the intrinsic Xase and prothrombinase complexes so as to maximise their enzymatic capabilities. Although the extrinsic pathway via TF is the main initiator of coagulation, it is the intrinsic pathway that has the greatest influence on clot propagation (88).

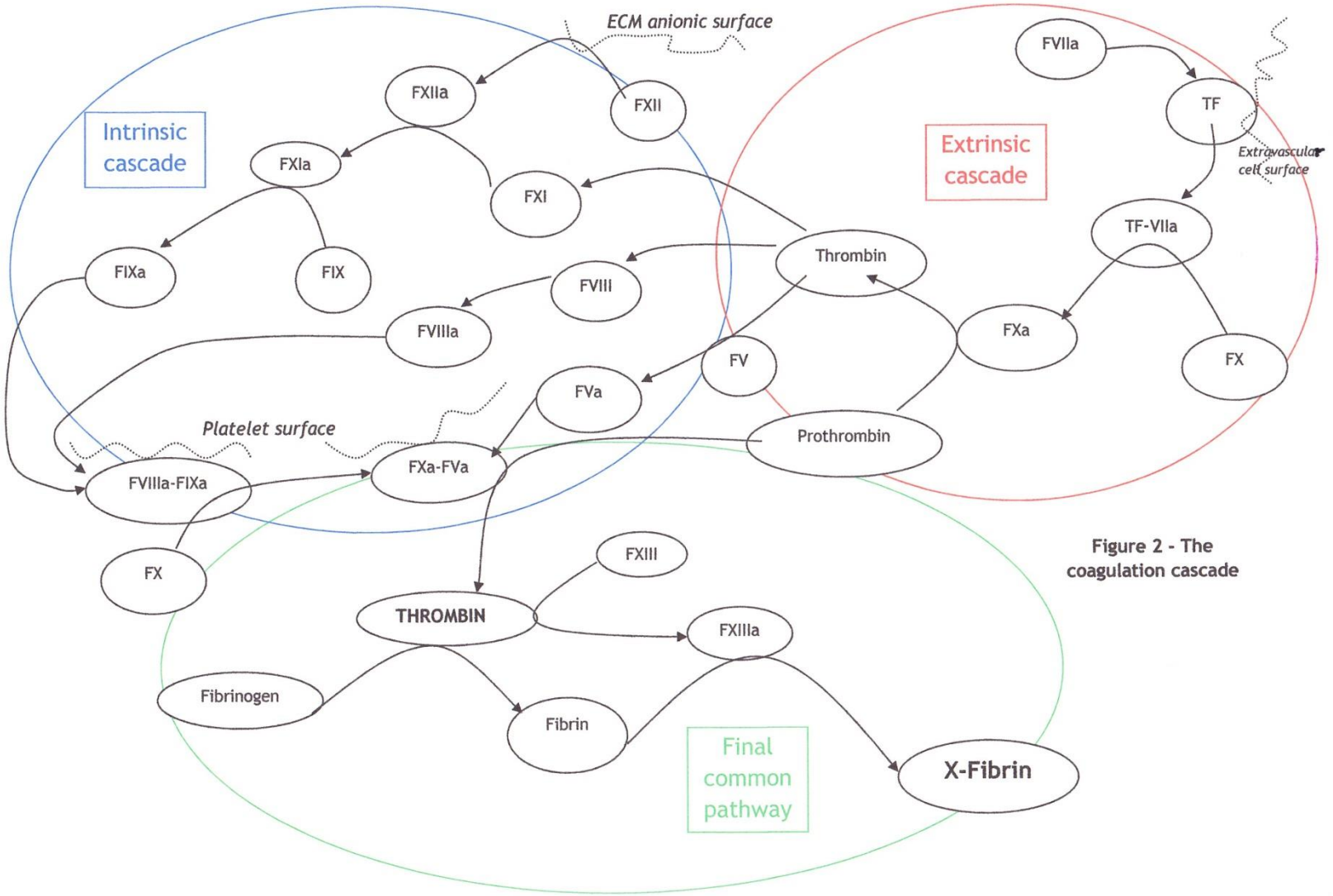


Figure 2 - The coagulation cascade

1.4.7 Control and termination of coagulation

Coagulation is a rapid process that requires regulation to prevent disseminated thrombus formation and damage to the vascular system. Various mechanisms contribute to the control of fibrinogenesis including the dilution of procoagulants in flowing blood, removal of activated factors by the reticuloendothelial system and the control of activated procoagulants and platelets by antithrombotic pathways. Antithrombotic pathways are anchored to vascular endothelial cells ensuring clot propagation is limited to areas of damaged endothelium.

1.4.8 Antithrombin (AT)

Antithrombin (AT) or antithrombin III is a serpin that circulates in plasma. It neutralises thrombin, FXa, FIXa, FVIIa-TF, FXIIa and FXIa by binding with them to form irreversible complexes. It has 2 functional sites, the reactive centre and a heparin binding site. The inactivation rate of AT is increased up to 10,000 fold by binding to heparins (86). Furthermore, heparan sulphate, a glycosaminoglycan found on endothelial surfaces, can also bind activated AT producing an endothelial surface coated in AT which mops up excess circulating coagulation factors (89).

1.4.9 Activated protein C (APC), protein S and thrombomodulin

Thrombomodulin is a transmembrane protein synthesised by and localised to endothelial cells that binds and inactivates thrombin. The thrombin-thrombomodulin complex activates protein C, then APC with its cofactor protein S inactivates FVa and FVIIa, and hence the intrinsic and extrinsic Xase complexes respectively. Protein S circulates in its free form or bound to C4b binding protein. In its free form it is a naturally occurring anticoagulant but is inactivated when bound to C4b (90). In the setting of inflammation, C4b is an acute phase reactant resulting in a reduction of free protein S, which may in part explain the pro-thrombotic nature of inflammatory states.

1.4.10 Tissue factor pathway inhibitor (TFPI)

TFPI is synthesised by endothelial cells. 90% of circulating TFPI is bound to low-density lipoproteins. TFPI directly inhibits FXa in a slow reaction then forms a complex with FXa and inhibits the TF-FVIIa complex. TFPI is the principal regulator of the initiation phase of thrombin generation (86). One of the effects of heparin is to release endothelium-bound TFPI into the circulation.

1.4.11 Fibrinolysis

Once endothelial repair is complete, the fibrin clot formed through coagulation is no longer required and must be removed to restore vessel patency and vascular function. Clot removal occurs through the process of fibrinolysis. Although described as discrete phenomena, fibrinogenesis and fibrinolysis occur simultaneously and regulate each other through inhibitory feedback. The fluctuation of fibrinolytic versus fibrinogenic factors and their inhibitory proteases determines which cascade dominates at any point in this process.

Fibrinolysis begins with the cleavage of plasminogen to plasmin by plasminogen activators. There are 2 types of plasminogen activator, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), the latter named for its abundance in urine (86). tPA has its main role in dispersing thrombus within the circulation whereas uPA has a more extravascular inflammatory role.

The activity of both tPA and uPA on plasminogen is relatively poor until fibrin is present when enzymatic activity is enhanced. Both tPA and plasminogen are adsorbed onto the surface of fibrin clot localising the production of plasmin to the site of clot and protecting it from inhibition by circulating α_2-antiplasmin. α_2-antiplasmin inactivates plasmin by irreversibly binding with it to form an inactive complex and in the circulation, rapidly mops up free plasmin. Within a clot, FXIIIa not only stabilises fibrin monomers but also incorporates α_2-antiplasmin into the clot structure. α_2-antiplasmin not only binds plasmin at the site of thrombus, but competes with plasminogen for binding sites on fibrin. However, plasminogen

concentration exceeds that of α_2-antithrombin therefore depletion will occur if plasmin continues to be generated and fibrinolysis will eventually occur (86).

1.4.12 Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 is the primary physiologic inhibitor of plasminogen activation in blood, targeting uPA and tPA. The major fraction of PAI-1 present in blood is from platelets where it is stored in α granules, but it is also synthesised in endothelial cells, smooth muscle cells and macrophages. Alongside its role as inhibitor of plasminogen activators, PAI-1 has a regulatory role for cell adhesion and migration (86).

1.4.13 Thrombin-activatable fibrinolysis inhibitor (TAFI)

TAFI circulates in the blood in complex with plasminogen and is activated by the thrombin-thrombomodulin complex. It cleaves plasminogen binding sites on fibrin clot thus reducing the efficacy of clot lysis by plasmin (86).

1.4.14 Fibrin(ogen) degradation products (FDPs)

A fibrinogen molecule is composed of 3 pairs of protein chains, (α, β and γ) in a dimeric structure bound via the γ chains. Thrombin cleaves a segment from each α and β chain resulting in the formation of fibrinopeptide A (FPA) and B (FPB) respectively. Thrombin cleavage of each fibrinogen molecule forms two FPAs, two FPBs and one fibrin monomer [see figure 3a]. Although adjacent fibrin monomers will polymerise to form a fibrin clot, the monomeric structure is less stable, and vulnerable to enzymatic breakdown by fibrinolysis. Crosslinking of adjacent D domains by FXIIIa increases structural integrity and reduces vulnerability to premature proteolysis (86) [see figure 3b].

When present in sufficient concentrations, plasmin can cleave polymerised fibrin, however, the crosslinked D domains remain joined and the adjacent E domains are cleaved [see figure 3b]. The resulting fibrin degradation products are one E fragment and one D-dimer. Plasmin can also degrade fibrinogen, and cleavage

occurs in an asymmetrical manner resulting in the formation of two D fragments and one E fragment, or intermediate breakdown products known as fragments X and Y (91).

The only unique fibrin degradation product is the D-dimer, as individual D domains and E domains may be the result of fibrin or fibrinogen breakdown. The presence of FPA and FPB indicate fibrinogen cleavage by thrombin has taken place but do not indicate successful fibrin clot formation. However, the presence of D-dimer not only indicates successful fibrin clot formation and conclusion of fibrinogenesis by FXIIIa crosslinking of fibrin monomers, but also subsequent fibrinolysis.

Figure 3a Fibrinogen and cleavage

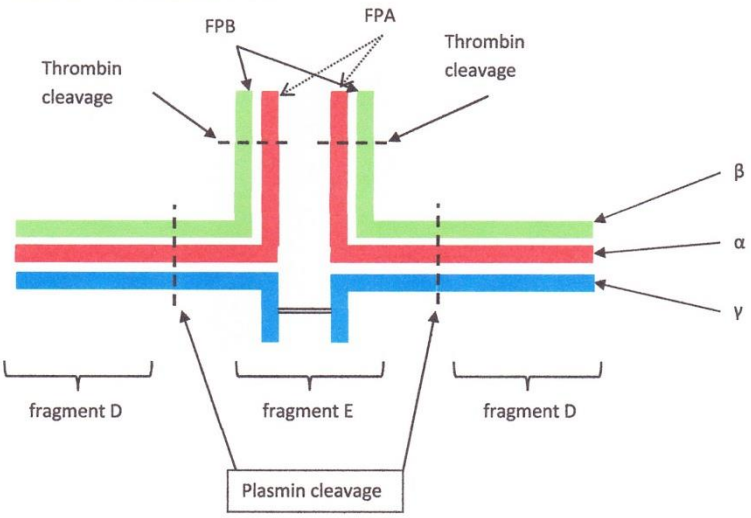
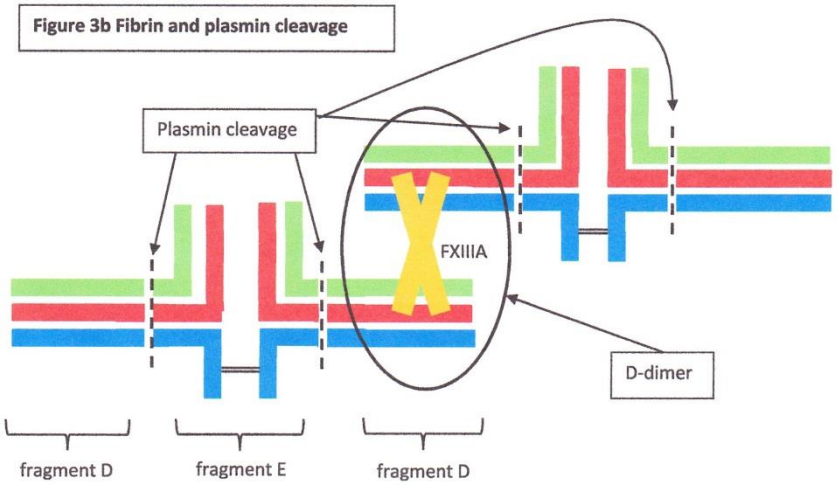


Figure 3b Fibrin and plasmin cleavage



1.5 Coagulation beyond haemostasis

Coagulation is traditionally taught as a mechanism for haemostasis, however it has wider roles in innate immunity and epithelial repair outside the vascular tree. In part, extravascular coagulation occurs due to plasma exudation in inflammatory states, however, extravascular tissues in some settings are capable of fibrinogenesis in the absence of plasma-derived clotting factors (92).

1.5.1 Innate immunity

The innate immune system consists of the complement system and cellular defences including, macrophages, neutrophils, eosinophils, mast cells and dendritic cells. The origins of innate immunity go hand-in-hand with coagulation, and clotting mechanisms may in fact be an evolutionary product of the innate immune system (93). As discussed above, coagulation is taught in terms of haemostasis, but if a fibrin clot is considered not only as a plug to prevent further bleeding but also a barrier to keep out injurious agents, many of its roles become clear. It is also no coincidence that the cells that play a central role in the pathogenesis of asthma derive from innate immunity. Furthermore, while the Th2 response key to atopy is part of adaptive immunity, it is the innate system that orchestrates and directs the balance between Th1/Th2 (94).

The fact that pathogens such as group A Streptococci and Staphylococci have developed strategies to disrupt and utilise coagulation to increase their pathogenicity further illustrates the link between coagulation and immunity. Group A Streptococci produce the plasminogen activator streptokinase enabling tissue invasion by fibrinolysis (95). The production of coagulase by Staphylococci results in fibrin formation and defence against phagocytosis and the production of staphylokinase, a plasminogen activator, reduces Staphylococcal virulence (96).

1.5.2 Protease-activated receptors (PARs)

Proteases such as those involved in coagulation function by cleavage of inactive zymogens which cascade as described above. These proteases have cellular actions beyond coagulation and it is the protease-activated receptors or PARs that enable these actions. PARs are transmembrane G protein-coupled receptors that carry their own tethered ligand that is revealed when proteolytic cleavage occurs (97). For example, thrombin activates lung fibroblasts to produce procollagen via PAR-1 (98) a mechanism that is involved in lung fibrosis. FXa can also stimulate fibroblasts to produce procollagen and proliferate (99).

Idiopathic pulmonary fibrosis (IPF) is a devastating condition with an average 2-3 year survival from diagnosis. In bronchoalveolar lavage fluid (BALF) specimens from patients with IPF, there is increased procoagulant activity and fibroblast survival via PAR-2 upregulation. Furthermore, in bleomycin-induced fibrosis, a model used to replicate IPF, PAR-2 knockout mice demonstrate reduced fibrosis, less myofibroblast differentiation and collagen expression is decreased (100). These mechanisms involving coagulation have implications for airway remodelling in asthma.

1.5.3 Acute lung injury/acute respiratory distress syndrome

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a spectrum of lung disease arising in response to local and systemic pulmonary insult resulting in rapid respiratory failure that carries a mortality of 40-60% (101). Increased capillary and epithelial permeability results in pulmonary oedema alongside increased pulmonary vascular resistance due to microemboli and microthrombosis, surfactant inactivation, loss of pulmonary compliance and ventilation/perfusion mismatch (102). The recognition that coagulation is activated in the lung in the condition is widespread and has been the subject of significant research.

Alveolar fibrin deposition occurs in ALI/ARDS resulting from enhanced coagulation and suppressed fibrinolysis (103). TF mediates enhanced intra-alveolar

fibrinogenesis in this setting (104), possibly stimulated by elevated levels of FVII activating protein (105). TF is increased in ALI/ARDS through plasma exudation and cellular sources including alveolar macrophages (106), neutrophils (107) and the alveolar epithelium itself (108). Mature neutrophils do not usually express TF (109), however stimulation with complement protein C5a or TNFα results in neutrophil expression of TF in this setting (107). Alveolar microparticles with TF activity are also present in ARDS, possibly of alveolar cell origin (108). Although TF is the major initiator of coagulation, clot propagation and production of thrombin in sufficient quantities to produce fibrin, requires the intrinsic cascade (88). Formation of the intrinsic Xase and prothrombinase complexes requires a plasma phospholipid surface, usually that of the platelet, however, platelets are not usually present in BALF (110). Plasma microparticles can serve this purpose (88), and the alveolar microparticles observed by Bastarache and colleagues (111) may take on this role in ALI/ARDS.

The suppression of fibrinolysis occurs in part due to a disproportionate elevation in TFPI when compared with elevated TF, but also inactivation of TFPI (112). Further fibrinolytic suppression occurs via the actions of PAI-1, α_2-antiplasmin and PAI-2 (103). Insufficient AT activity is also implicated, alongside reduced APC (102). APC exhibits anticoagulant effects by inactivation of FVa and FVIIIa, alongside anti-inflammatory actions and stabilisation of endothelial barrier permeability via PAR-1 and endothelial cell protein C receptor (113). The PROWESS study has shown that intravenous APC in severe sepsis can reduce mortality (114) but this benefit has not been shown in ALI/ARDS (115).

A variety of other therapeutic strategies have been investigated in ALI/ARDS including animal studies of intravenous (116) and nebulised (117) heparin which showed promise with nebulised therapy reducing lung oedema, cast formation and cellular infiltrates but no benefit when intravenous heparin was used. Phase 1 human studies of nebulised heparin have demonstrated practical application and a lack of serious adverse events but further study is required for this therapy (118).

β_2 receptor agonists show potential benefit in this setting acting via bronchodilatation, increasing clearance of lung oedema and anti-inflammatory effects but randomised controlled studies investigating β_2 agonists in this area are lacking (119). The antioxidant N-acetylcysteine (NAC) does not confer benefit in established ALI/ARDS (120). Attempts to increase fibrinolysis using uPA inhalation in animal studies demonstrated increased alveolar fibrinolysis (121) and phase 1 human studies of plasminogen activator therapy in humans show a lack of significant side effects (122). Finally, inhibition of TF-mediated coagulation has demonstrated mixed results. Active site inactivated recombinant FVIIa showed increased mortality against placebo and a trend to increased risk of serious bleeding as doses increased (123). Using anti-TF antibodies, phase I trials demonstrated safety using intravenous administration (124).

1.6 Coagulation in asthma

There has been increased focus on the role of coagulation in asthma in recent years. Evidence from post mortem examination of the lungs of a patient who died of status asthmaticus shows fibrin deposition along the distal airways and alveoli (125). Murine models of airway inflammation confirm this deposition of fibrin and demonstrate increased AHR with administration of nebulised fibrinogen and thrombin that is reduced with administration of the fibrinolytic initiator uPA (125). PAI-1 is a major suppressor of fibrinolysis and increased fibrinolysis by either increased uPA or decreased PAI-1 has been shown to reduce AHR in mouse models. Furthermore, the pro-fibrotic chemokine TGF-β1 increases bronchial expression of PAI-1 and the resulting airway remodelling may be reduced by uPA administration (126). Plasminogen itself increases leukocyte recruitment into the airways in murine models (127). A reduction in FVII levels in mice results in decreased AHR, airway remodelling and eosinophilic airway inflammation (128), and increased FXa expression is observed in airway inflammation and is again linked to airway remodelling (129).

Plasma exudation resulting from inflammation in asthma carries with it coagulation factors. However, in cell culture models of normal human bronchial epithelium (NHBE) subjected to mechanical scrape damage, a TF-dependent repair response has been observed that occurs in the absence of plasma (92). Conversely, NHBE cell cultures subjected to compressive stress such as that which occurs during bronchospasm, promotes a fibrinolytic response (130). This increase in fibrinolysis could represent increased epithelial damage and defective repair, however, attenuated wound repair has been observed in NHBE cell cultures by blocking the uPA-uPA receptor (uPAR) interaction (131), demonstrating fibrinolysis as well as fibrinogenesis is necessary for efficient epithelial wound repair.

Human studies in asthma reveal increased thrombin generation in the airways that may be linked to airway remodelling (132). Bronchial allergen challenge activates airway coagulation independently of leukocyte infiltration (133). BALF after allergen challenge demonstrates increased thrombin activity, and this BALF exhibits mitogenic activity when exposed to cultured human fibroblasts, which is suppressed by hirudin, a specific thrombin inhibitor (134). Platelets are a source of numerous cytokines and coagulation factors, and are activated in the asthmatic airway after allergen challenge (135). During asthma exacerbation, higher levels of platelet activation are observed when compared with healthy controls, an effect that increases with steroid treatment (136).

When examining clinical asthma subgroups, in stable severe asthma, an exudative pro-fibrinogenic and anti-fibrinolytic environment occurs despite treatment. However, in moderate treated asthma, the balance slightly favours fibrinolysis akin to the normal airway, but if inhaled steroid treatment is weaned in this group, the balance increases in favour of fibrinolysis (137). These observations suggest that; in milder forms of the disease inhaled steroids optimise epithelial repair; but in more severe disease excessive fibrinogenesis results in increased AHR and remodelling as seen in murine models (125). The importance of the balance between fibrinogenesis and fibrinolysis is reflected in the observation that while uPA and

PAI-1 are both increased in asthmatic sputum compared with healthy controls, and both rise in response to allergen challenge, the ratio of uPA:PAI-1 falls (138). This is further reflected when examining TAFI, a suppressor of fibrinolysis. Murine models of airway inflammation demonstrate that the presence of TAFI reduces AHR and airway inflammation, an observation which contrasts the effect of PAI-1. Furthermore, a reduction in TAFI levels promotes airway remodelling, possibly due to compensatory increased PAI-1 (139), highlighting the complex interplay between the fibrinogenic and fibrinolytic pathways.

Finally, when studying moderate stable asthma before and after weaning inhaled corticosteroids, Brims and colleagues (80) observed a marked shift towards a pro-fibrinogenic environment within the airways of a patient 5 days prior to an exacerbation. Of particular note, the inflammatory changes occurred without any detectable deterioration in conventional clinical parameters such as spirometry or FeNO, and before any symptoms of exacerbation were present.

1.7 Hypothesis

Given the potential importance of the coagulation cascade in acute respiratory disease states, the following hypothesis was tested:

Markers of fibrin turnover are raised in plasma during exacerbations of asthma that improve with recovery; and their presence in urine predicts the onset of a new exacerbation in otherwise stable asthma.

2.0 METHODS

2.1 Study design

This is a cohort study in two parts comparing markers of fibrin turnover during asthma exacerbation, with the recovery state. Part 1 recruited patients requiring treatment in hospital for acute exacerbation of asthma and comparison was made at recovery. Part 2 prospectively recruited patients with moderate and severe asthma when well, and observed them until asthma exacerbation, and through to subsequent recovery. A study period of two years was proposed with parts 1 and 2 recruiting in parallel. Target recruitment was 100 study subjects for Part 1, and 40 subjects for Part 2, 20 with moderate and 20 with severe disease defined by baseline steroid dose and GINA criteria (appendix 2).

The full study protocol is available in appendix 3, and details of study procedures will be outlined in relevant sections. In brief, study activity included clinical and spirometric assessment at each study visit alongside measurement of FeNO, and urine and blood sampling. Sputum induction and full pulmonary function tests (PFTs) were performed on selected participants. Skin prick testing for sensitivity to common aeroallergens was performed at one study visit. Participants were asked to record PEFR and symptom diaries between visits. Part 2 participants were also asked to collect thrice weekly urine specimens.

2.2 Approvals

A favourable ethical opinion was granted by the Berkshire Research Ethics Committee (REC) on 12th August 2010 (REC reference 10/H0505/59 see appendix 4), and local research governance approval was granted for recruitment to commence on 23rd September 2010 by Portsmouth Hospitals NHS Trust (PHT) acting as study sponsor (appendix 5).

During the course of the study period two substantial amendments to the study protocol and 1 minor amendment were submitted to the Berkshire REC and

received approval. These amendments related to study recruitment activity and are detailed in the relevant sections below.

2.3 Sample size/power

Based on published studies (137), a 2 to 72-fold increase in pre-exacerbation levels of coagulation factors was observed in sputum compared to stable mild to moderate disease, and similar differences were expected in blood and urine. Simulations were performed after logarithmic transformation at 90% power (5% significance) to detect between 1 to 5 fold differences in coagulation factors. Based on these calculations, 100 subjects for Part 1 would provide sufficient power to detect these differences. Anticipating potential subgroup analyses, interim analysis was planned after recruiting 50 subjects to establish whether 100 recruits in total would be sufficient for comparison between subgroups. The interim analysis would also provide useful preliminary data to guide any necessary sample size increase for Part 2. In June 2011 after recruiting 50 subjects, statistical advice was sought regarding this interim analysis and although not formally recorded, it was estimated that nearly 300 participants, 100 per subgroup would be needed for subgroup comparisons. The study team acknowledged this figure but with a maximum of one further year of study recruitment and 50 participants so far recruited in the preceding 10 months, this figure was most likely unattainable.

The prospective Part 2 study is pilot work and the numbers based on experience of intensive prospective follow up of adults with asthma over about 4 months per subject. Standard tests of significance (parametric and non-parametric) were used to compare between exacerbation and recovery time points. Analysis of variance (ANOVA) was used to examine exacerbations preceded by a rise in coagulation factors compared to those that are not. A minimum of 12 patients in each sub-group (moderate and severe) were required for useful ANOVA analysis, and recruitment of 40 subjects (20 moderate and 20 severe) was chosen to allow for subject withdrawal and failure to exacerbate.

The details of statistical tests used to analyse specific data are presented alongside individual results in Chapter 3 and Chapter 4 of this thesis. All statistical analyses of study results were performed using Stata version 12.1 (Statacorp LP, Texas, USA) by Paul Bassett of Statsconsultancy (Amersham, UK).

2.4 Study subjects

Written informed consent was obtained from all individuals recruited prior to commencement of any study activity (appendices 6, 7).

2.4.1 Inclusion criteria

Both Parts 1 and 2 of the study recruited "real life" patients with asthma without prior alteration of medications. This strategy provides real strength to our results in terms of translating our findings to a patient population.

For both study arms:

- A diagnosis of asthma >1 year
- Age 18-70 years

Specific to Part 1:

- An acute exacerbation of asthma requiring admission to hospital for treatment of that exacerbation

Specific to Part 2:

- Moderate asthma - chronic moderate persistent disease (appendix 2) requiring medium daily doses of inhaled corticosteroids (>500-1000mcg of BDP or equivalent (appendix 1))
- Severe asthma – severe persistent disease (appendix 2) requiring high dose inhaled corticosteroids (>1000-2000mcg BDP or equivalent(appendix 1)) and/or oral corticosteroid use

2.4.2 Exclusion criteria

- Current or ex-smokers with >20 pack year history
- Significant cardiopulmonary co-morbidities
- Clinically significant neurological, renal, endocrine, gastrointestinal, hepatic or haematological abnormalities uncontrolled with standard treatment.
- Regular use of an anticoagulant
- Bleeding diathesis
- Alcohol and recreational drug abuse
- Treatment with other immunomodulators
- Concurrent participation in another interventional study
- Abnormal chest X-ray (CXR)
- For Part 2: an asthma exacerbation in the 4 weeks prior to study recruitment (visit 1)

For the purposes of an asthma diagnosis, prior physician diagnosis was accepted as sufficient evidence, and bronchial hyperreactivity testing was not undertaken in line with the majority of "real-life" practice. In terms of other airway disease that may mimic asthma, the most likely condition would be chronic obstructive pulmonary disease (COPD). In a UK population COPD is almost entirely due to smoking so an exclusion cut off was initially set at 10 pack years (1 pack year = 20 cigarettes per day for 1 year or equivalent). This was increased in a protocol amendment to a maximum of 20 pack years, as initial recruitment for Part 1 found a higher than anticipated pack year history amongst potential study recruits. However, for those participants with between 10 and 20 pack years, full pulmonary function testing was performed to look for evidence of COPD other than airways obstruction such as a reduction in gas transfer.

For Part 1 study recruits, hospital admission for treatment included those admitted to a ward, those seen in the Emergency Department (ED) who sufficiently responded to treatment to be safely discharged, and patients treated on a similar basis at the Respiratory Centre at Queen Alexandra Hospital (QAH).

In Part 2 subjects who did not require admission to hospital for their exacerbations, a chest X-ray was not performed to exclude other acute confounding co-morbidity (e.g. pneumonia) unless clinically suspected.

The exclusion criterion of an abnormal CXR was designed to exclude acute inflammatory conditions such as pneumonia, or obvious diffuse parenchymal lung disease that may influence markers of fibrin turnover (98). It is worthy of note that the term "abnormal CXR" did not clearly distinguish which factors were true exclusions, and the research team used clinical discretion in a few cases, for example, one case of minimal apical scarring due to tuberculosis (TB) as a child was not excluded as this was deemed not to be as significant confounding factor.

Although not specifically stated, pregnancy was also an exclusion criteria as this is known to be a pro-thrombotic state as illustrated by high plasma D-dimer levels (140).

2.4.3 Methods of recruitment

In order to maximise the potential for achieving recruitment targets within the proposed timescale, two main study sites were identified, QAH/PHT and Basingstoke and North Hampshire NHS Foundation Trust (BNHFT).

Part 1:

For the acute admission arm, a daily search of PHT computer admission records was performed to identify patients admitted to QAH because of their asthma. This was supported by posters (appendix 8) in the ED, Medical Assessment Unit (MAU) and respiratory wards at QAH inviting medical teams to contact the study team if they

identify patients admitted with acute asthma. Respiratory Centre staff also visited the above clinical areas to liaise with clinicians and identify potential recruits. Patients identified as having an exacerbation during difficult asthma clinic visits at QAH, or patients attending The Respiratory Centre at QAH for treatment of an exacerbation were also invited to participate.

At BNHFT, clinical teams highlighted potential recruits to Dr Sal Matti, site Principal Investigator (PI) who contacted Dr Owen who undertook study recruitment activity.

Recruitment to Part 1 was relatively successful and 55% of those screened were recruited to the study. This does not, however, account for a suspected significant number of potential recruits who were not screened. Active recruitment was limited to 1 research fellow and 1 research nurse with the chief investigator covering for periods when the research fellow was unavailable. Furthermore, study recruitment was essentially limited to the hours of 9am to 5pm Monday to Friday in line with the hours of work of the study team. For those patients who attended ED but were discharged home without being referred to the admitting medical team it was hoped that the study team would be contacted but very few patients were identified and all of these patients declined to return to the hospital for screening when contacted. Similar confounding factors were experienced on the MAU whereby the study team were very infrequently contacted regarding patients. Overall this was less of a problem as the study team were able to attend the MAU twice a day (to give potential patients an opportunity to participate in the research), and computerised admission records present in this area, allowed eligible recruits to be more readily identified. Furthermore, the national pressures to admit or discharge patients within 4 hours that apply for the ED do not apply once a patient is admitted to MAU, allowing more time to address such patients.

In terms of improving recruitment to Part 1, the key factors identified would be to have more staff cover after 5pm and also at weekends. Within the ED, adhering to the time constraints upon clinical staff and maintaining patient throughput is paramount to ensure congestion and queuing of ambulances doesn't occur. While

not unique to QAH, this was a particular problem during the study period in part due to the physical layout and staffing levels in the ED. In order to relieve some of these pressures, the ability for the research team to take patients to a separate area to complete study activity would enhance recruitment.

Study recruitment did take place at BNHFT, however, this was dependent on patients being identified primarily by Dr Sal Matti, consultant respiratory physician who then contacted Dr Owen who travelled to BNHFT and performed screening and study activity. This method of recruitment was entirely dependent on 1 individual to identify potential recruits and then on the availability of Dr Owen on a particular day. Despite 100% of those screened at BNHFT being recruited, the total number was only 3 participants (4%) and the logistical constraints were considerable confounding factors. A dedicated recruiting team at each site should be considered if the study were to be repeated.

The initial target for recruitment to Part 1 was 100 study participants which was not reached during the research fellowship. Dr Owen was granted a period of two and a half years out of his Wessex respiratory medicine training rotation for the purposes of undertaking a postgraduate research project. This period finished in July 2012 so the decision was taken by the study team to cease recruitment activity by May 2012 to enable Dr Owen to complete the laboratory analyses prior to returning to full time clinical work.

Part 2:

Prospective study activity was limited to PHT for logistical reasons. The difficult asthma clinic database was searched for potential recruits who were invited to participate by letter (appendix 9), or approached during routine clinic visits. Generic advertisements were placed in the local newspaper (The Portsmouth News) regarding respiratory research at QAH inviting interested people to make contact with the Respiratory Research Team. Dr Owen was interviewed regarding respiratory research by local hospital radio and a further generic advert was placed on the PHT staff intranet inviting staff to contact the Respiratory Centre.

Local GP surgeries (Brook Lane Surgery in Locks Heath and Portsdown Group Practice in Cosham) were employed as Patient Identification Centres (PIC). Surgery staff searched practice databases for potential recruits, then contacted patients seeking consent to send them a study Participant Information Sheet (PIS) with contact details of the Respiratory Centre if they wished to be seen for study recruitment (see appendix 10 for surgery invite letter).

Initially it was expected that the majority of the recruits would be identified from the asthma database at QAH compiled in previous years from the local population attending the Respiratory Centre. However, many of these patients were excluded in pre-screening from patient records due to factors such as cigarette consumption and immunomodulatory therapy. Despite letters sent directly to patients (appendix 9), response numbers were poor. The design of the letters may have impacted on this and different approaches such as email or text message might be considered in the future in line with the cultural shift away from traditional written communication. Although generic adverts for QAH research activity in local newspapers and on local radio were made, ethical approval for advertising specific to this study was not sought. It is possible that a more specific advert for the study would have improved feedback. It is also worth noting that those who did respond to the generic adverts had very mild asthma that was outwith the study inclusion criteria.

There was particular difficulty in identifying patients with moderate asthma in terms of inhaled steroid dose. When recruitment to Part 2 began, those patients contacting the research team through adverts were too mild and those identified from outpatient clinics and The Respiratory Centre database tended to be too severe. It was anticipated that a significant proportion of patients with moderate asthma would be able to be recruited in line with previous work by our group (80, 137), which was based on the GINA 2008 guidelines (7) which is unchanged in the latest iteration of GINA guidance published in 2014 (5). In order to identify this population, a study amendment was submitted and approved to utilise local GP surgeries as PIC sites. Although this strategy did identify 6 participants (10% of

those contacted), strict protocols were imposed by the approving body (Primary Care Research Network (PCRN)) detailing the methods to be employed for identifying patients. Research staff were not allowed access to patient records within surgeries and surgeries were required to contact patients asking their permission to be sent the study invitation letter (appendix 9). Research staff were only allowed to contact the patient after the patient had first contacted the research team. Despite funds being provided by the PCRN to PIC sites, no staffing resources were provided to search databases and make contact with patients.

The numerous steps involved in this strategy limited its success alongside resourcing limitations, particularly in terms of staff. Future consideration should again be given towards email contact with patients and resourcing in terms of PCRN or community staff with more time and more access to the relevant details pertaining to the study who can research pseudo-anonymised patient records.

In broad terms, it is difficult to explain why there were not more patients who fell into this group. Knowing the inhaled steroids available for prescription, it is plausible that some patients will fall below the 500 mcg BDP threshold and some above the 1000 mcg. There should, however, be a significant proportion receiving budesonide 800mcg or QVAR 400mcg (equivalent to 800 mcg BDP). Local prescribing patterns may have an influence on the apparent paucity of these patients. Equally this could be explained by this group being relatively well controlled and not requiring secondary care services, hence them being unknown to the hospital's service. Furthermore, if well controlled, such patients may be less inclined to take part in research. It is worth noting that of the 10 patients who did respond, 7 were too mild to be included which reflects inaccuracy in the records of the GP surgeries that were screened, or a failure by surgery staff to identify the correct target group. Once the patient had received the invitation letter which clarifies the necessary steroid dose, they may have recognised themselves that they did not meet the inclusion criteria and hence did not respond.

Both study arms:

Dr Owen and Prof Chauhan, as part of educational presentations regarding asthma within PHT, invited clinicians to identify potential research patients to the Respiratory Centre at QAH.

Once patients had been highlighted to the study team, they were approached and given the relevant PIS for each study arm (appendices 11, 12).

2.4.4 Screening

Part 1:

Once potential recruits were identified by staff, pre-screening of admission records was performed to identify any clear documented exclusion criteria such as past medical history (PMHx), anticoagulant medication, or concurrent admission diagnosis (e.g. pneumonia). If none were identified, patients were approached by study personnel and given a PIS then left for at least half an hour to consider participation. If patients wished to participate, written informed consent was obtained and screening was completed by checking all inclusion/exclusion criteria. Baseline assessment was performed at this initial screening visit and is detailed below.

Part 2:

After identification, potential participants were given a PIS if agreeable, and after at least a day to consider taking part, an appointment was made for screening and initial assessment which took place at the same visit.

After successful recruitment in both study arms, a letter was sent to the participant's GP informing them of their patient's involvement in the study (appendix 13) along with the relevant PIS.

2.4.5 Study visit schedule

Study visit schedules are outlined for Parts 1 and 2 in figures 4 & 5 respectively. Participants were offered a second follow up visit if not recovered at the first follow up. Participation completed at the second follow up whether recovery was achieved or not.

Figure 4 – Part 1 study visit schedule

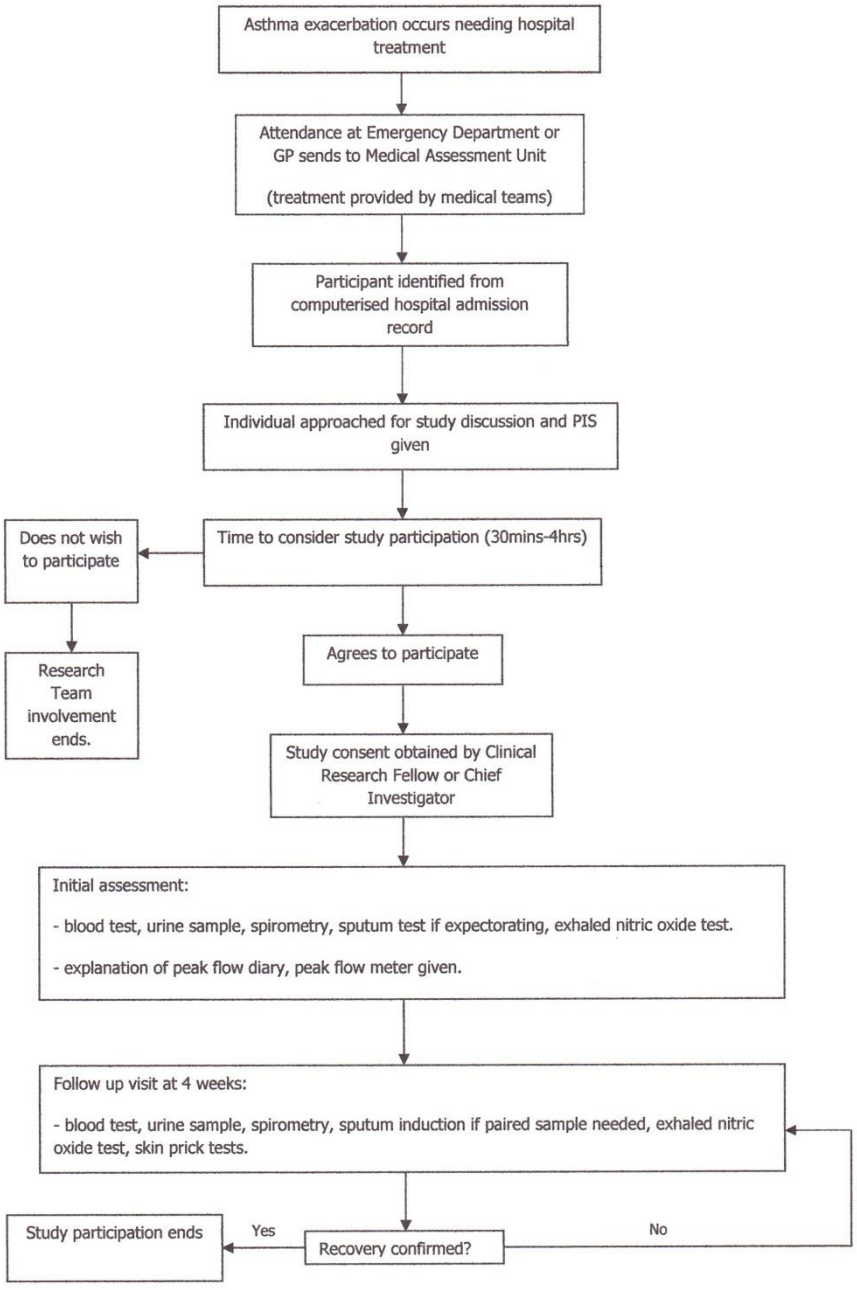
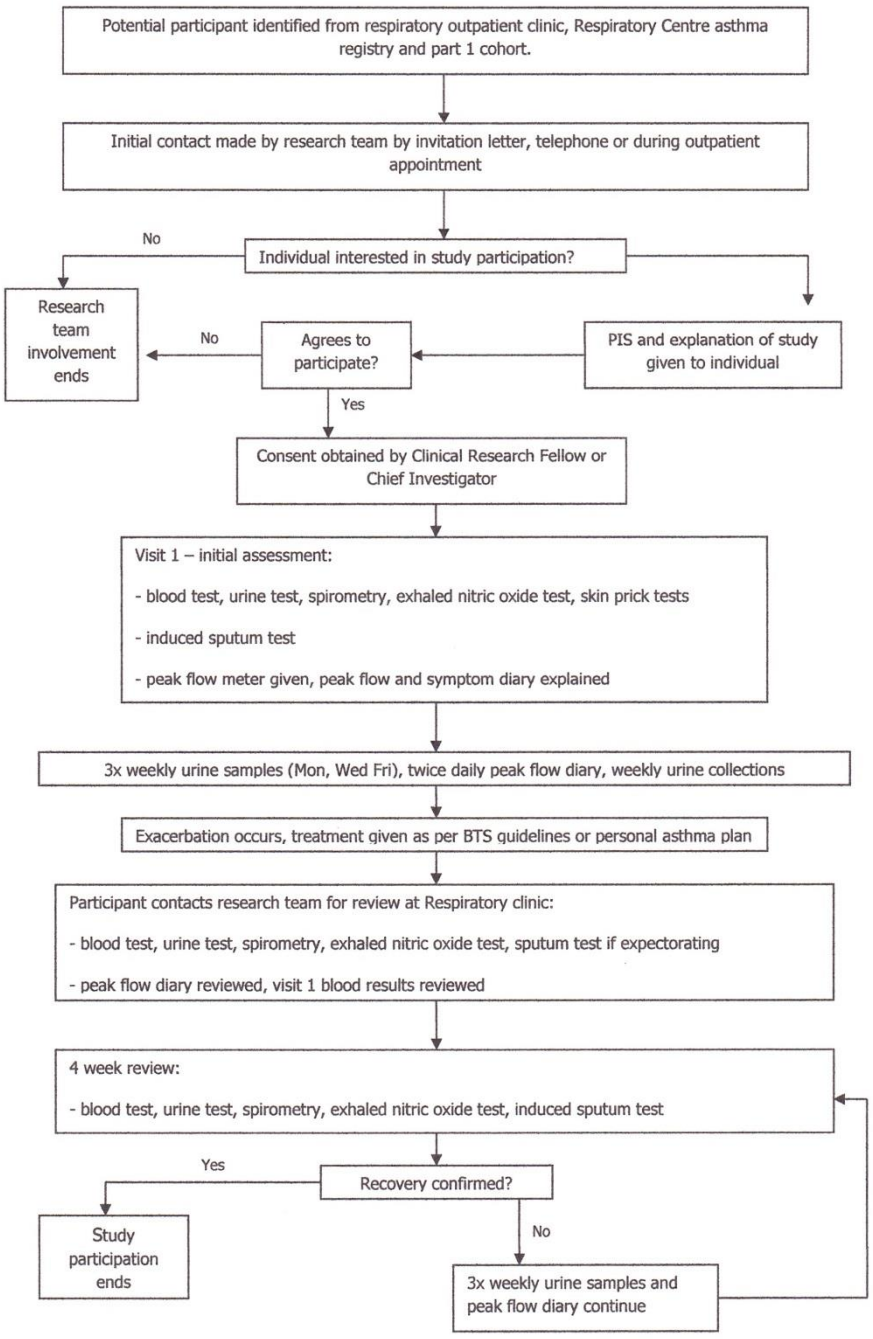


Figure 5 – Part 2 study visit schedule



2.4.6 Study follow up

Overall, the rate of participants lost to follow up was small, 15 of 84 (18%) for Part 1 and none in Part 2.

In Part 1, a follow up appointment was planned for 4 weeks after admission to assess recovery, then a further follow up was offered at 8 weeks if recovery was not achieved at follow up 1. Part 2 was designed to mirror this model after the exacerbation visit. The follow up visit was scheduled at the time of exacerbation in both Parts 1 and 2 of the study. The fact that patients were being given a date for follow up during an acute admission, may in part explain why failure to attend appointments occurred, for example, patients not having access to diaries or schedules. Several of the patients had follow up appointments made with clinical teams in line with national guidelines (4). This may have added a degree of confusion in that some patients were unclear that they were due to attend both appointments and some may have been unwilling to attend more than one appointment due to other commitments. The patients were not given a specific appointment card or sent a reminder letter for this research appointment which could also have impacted on their attendance. Failure to attend follow up was not a problem in Part 2 of the study, but this is likely to reflect a selection bias as those patients who had committed to Part 2 were already regularly collecting samples and had attended an outpatient appointment at baseline. Although the offer was made to reimburse travel costs for study participants, very few recruits made this request. No other incentive was offered to patients when approached regarding study enrolment which could be a consideration to increase both initial recruitment and subsequent follow up attendance. Whether or not financial incentives should be offered over and above reimbursing costs is reviewed by Draper and colleagues (141) and remains a matter of debate.

It is worth noting that although follow up was planned to be for 4 and 8 weeks post-exacerbation, in Part 1 first follow up occurred on average 35 days (5 weeks) with a range of 28-91 days, and second follow up 65 days (9 weeks 2 days) with a range of 41-96 days. In Part 2 exacerbation to first follow up average was 30 days (4 weeks 2

days) with a range of 28-37 days, and exacerbation to second follow up 61 days (8 weeks 5 days) with a range of 56-70 days.

The variation in duration between visits is due to factors discussed previously including, staffing levels, lack of appointment reminders, confusion between clinical and research follow up, but also due to flexibility on the part of the study team in order to work around patient's availability. Future studies should consider a more formal arrangement for scheduling appointments and a reminder service, possibly in writing but also considering text or email contact. In terms of comparable results, a more rigid window in which to schedule appointments should be considered, for example follow up 1 should be arranged at 28 days +/- 4 days and follow up 2 56 days +/- 4 days.

The time from asthma exacerbation to clinical recovery and resolution of acute inflammation will be discussed in more detail below.

2.4.7 Study documents and data recording

A number of documents were designed and approved for use in the study by the Berkshire REC, copies of which are contained in appendices 3-19. Paper documentation was used primarily due to study budget but had the additional advantage of being transferable across study sites without the need for using different information technology (IT) systems and the potential data protection consideration involved. In broad terms the study documentation was successful in recording a comprehensive set of study data.

During the study, further improvements were made to the CRF to facilitate improved data recording that included changes in the layout, and addition of the GINA 2008 disease severity criteria. There were other areas where participants failed to record some data. For example, the admission study activity for Part 1 took place as close to the day of admission as possible, however, participants could remain an inpatient for several days then retrospective recording of medication changes and duration was required by reviewing casenotes which were not readily available or always clearly accurate. This was also apparent when calculating

durations of drugs such as oral corticosteroids or antibiotics at follow up, which relied heavily on patient recollection. Although the PEFR and symptom diary had areas for this to be recorded, some participants failed to record information or forgot to return their diary.

2.5 Study visit activity

2.5.1 Clinical parameters

At each study visit a clinical history was taken and physical examination including blood pressure, pulse, oximetry and respiratory examination was performed. CRFs were used to record study visits and are contained in appendices 14 and 15. Key asthma symptoms (breathlessness, cough, wheeze, chest tightness, night wakening, chest pain and sputum production) were recorded alongside reliever use per twenty-four hour period, and a composite score of symptomatology was obtained at each time point.

2.5.2 Spirometry

Spirometry was measured at every visit. Where possible, three consistent measurements were taken in line with ATS/ERS guidelines (142). However, investigator discretion was allowed when symptoms such as cough occurred, especially at exacerbation visits.

Two handheld spirometers were used throughout the study period, the first a Microlab 3500 (Carefusion, Hampshire, UK) and the second a Vitalograph Alpha Touch (Vitalograph, Buckinghamshire, UK). Regular calibration of the machines was performed as per manufacturer's instructions. The European Community of Coal and Steel (ECCS)/ERS 1993 (143) reference values for normal range were used. Individual study participants used only one machine for all of their spirometric measurements allowing accurate intra-individual comparison between study visits.

2.5.3 Exhaled nitric oxide

FeNO was measured at all study visits (except when too breathless at exacerbation) using a NIOX MINO machine (Aerocrine, Solna, Sweden). Two machines were used for this test, the NIOX MINO 03-1000 and the NIOX MINO 09-1000 as per manufacturer's instructions. Both are self-calibrating and according to the manufacturer, results from each machine are interchangeable as both devices use the same internal sensor.

2.5.4 Skin prick testing

Skin prick testing (SPT) for sensitisation to common aeroallergens was performed on all study participants with the exception of those who were unable to stop antihistamines on clinical grounds, in these circumstances consent was obtained to use any previous available results. SPT was performed at follow up for Part 1 and at visit 1 for Part 2.

The panel of allergens tested consisted of positive and negative controls, tree pollen, grass pollen, house dust mite, cat epithelia, dog and Aspergillus fumigatus. Allergens were applied to the forearm and introduced into the skin using a lancet, and excess allergen was removed by blotting with a tissue, taking care not to cross-contaminate allergens. In line with existing PHT practice, results were read at 15 minutes and measured in millimetres at the widest point of any weal. A positive result was regarded as a weal 3mm greater than that of negative control. Allergens were purchased from Diagenics Ltd (Milton Keynes, UK) and are detailed in table 1 below.

Table 1 – Skin prick test reagents used			
Number	Reagent name	Constituents	Concentration
Allergopharma D-21462			
901	Negative control	Glycero-saline	n/a
902	Positive control	Histamine dihydrochloride	1.7mg/ml
013	Tree mix, mid blossoming	n/a	100000 BU/ml
306	Dog	n/a	10000 BU/ml
401	Aspergillus fumigatus	n/a	10000 BU/ml
725	House dust mite	Dermatophagoides pteronyssinus	50000 SBU/ml
Merck skin testing solution			
006	Grass mix	n/a	50000 SBU/ml
309	Cat epithelia	n/a	50000 SBU/ml
n/a = not available, SBU = Standardised Biological Units, BU = Biological Units			

2.5.5 Pulmonary function testing

In terms of this study, full PFTs refers to the combination of dynamic and static lung volumes and gas transfer. Although spirometry is routinely measured as part of this test at PHT, the results reported for spirometry in this study were taken from the isolated spirometric analysis described above to enable comparison between study visits. Both body plethysmography and helium dilution methods were employed for lung volume and gas transfer evaluation.

Full PFTs were performed on all Part 2 recruits at visit 1. For Part 1, PFTs were only performed at follow up if smoking history exceeded 10 pack years (1 year smoking 20 cigarettes per day = 1 pack year). This was to exclude confounding smoking-related airways disease such as COPD. Usual medications were not withheld prior to measuring PFTs. PFTs were measured as per PHT protocols by the Respiratory Physiology Department using nSpire ZAN 310 FRC-He and ZAN 500 Body USB machines (nSpire Health Ltd, Hertfordshire, UK) in line with manufacturer's instructions. PHT employs the Association for Respiratory Technology and Physiology (ARTP)/BTS testing protocol for measurement of lung volumes and gas transfer (143). Normal reference values used were ECCS (143).

2.5.6 Sputum induction

Sputum induction and analysis was intended to allow inflammatory classification of study participants. Furthermore, the examination of markers of fibrin turnover in sputum during asthma exacerbation was planned. Although such markers have been investigated in stable asthma (137) and viral-induced models (144), only fibrinogen has previously been investigated during acute asthma exacerbation in sputum (145).

Specific written informed consent was obtained from study participants after receiving written information regarding the procedure (appendices 16, 17).

Sputum induction was performed in line with PHT protocols and documented on a protocol worksheet (appendix 18). Standard protocol was employed for the majority of participants, and a second protocol used for those deemed at high risk of bronchoconstriction. The factors used to determine those at risk of bronchoconstriction are as follows:

- Known brittle asthma
- Low baseline FEV_1 (<1 litre) or FEV_1 <50% predicted
- Previous bronchoconstriction with nebulised saline
- Development of significant symptoms during induction
- Participant request
- Investigator discretion

2.5 mg nebulised salbutamol was given prior to commencing sputum induction and FEV_1 was measured (see spirometry, section 2.5.2) at baseline for both protocols. FEV_1 was measured at variable intervals throughout the procedure depending on the protocol employed. Nebulised saline at differing concentrations (0.9 to 5%) was administered via a DeVILBISS Ultraneb (Sunrise Medical Ltd, West Midlands, UK)

according to manufacturer's instructions for the purposes of sputum induction. For FEV_1 measurement intervals and saline concentration schedule please refer to appendix 18. Prior to study commencement but after completion of study documents, trust clinical protocols for sputum induction were changed using 5% saline as the standard alternative to 4.5% saline and this was also adopted for study purposes.

Acute bronchospasm is a recognised complication of sputum induction (146) and during study design, it was felt that attempted sputum induction during acute asthma exacerbation posed unacceptable risk to participants of worsening that exacerbation. However, it was anticipated that a significant proportion of patients during exacerbation would expectorate sputum, enabling comparison with sputum induced at recovery for both study arms, and also at baseline in Part 2. Where participants did not expectorate sputum during exacerbation, the lack of a paired comparative sample made sputum induction at recovery unnecessary, and equally when sputum induction in Part 2 was unsuccessful at baseline, further induction attempts in these participants was not attempted.

The number of participants expectorating sputum was unexpectedly low, only 19 of 82 (23%). As far as we are aware, the incidence of spontaneous sputum expectoration at the time of asthma exacerbation has not previously been published. Anecdotally, the clinicians involved in the study had anticipated nearer half of the subjects would expectorate sputum during exacerbation due to the acute inflammation that occurs. For reasons that included baseline severity of asthma and spontaneous expectoration at follow up, only 8 sputum inductions were attempted in Part 1 and in Part 2, a further 14 were made. Of the 22 sputum inductions attempted during the study, 13 (59%) were successful in producing a sputum sample. Other studies report a success of 80-90% (6). The protocol used in our study was in line with international guidelines (147), and the exact reasons for our lower success are unclear.

There is emerging evidence that sputum induction is safe even during acute severe exacerbation of asthma (145, 148, 149) and this should be taken into consideration for future study design. Due to the overall paucity of samples, sputum was not examined as part of this study.

2.5.7 Peak flow and symptom diary

PEFR was recorded at each study visit. Participants were also asked to complete a symptom and PEFR diary between study visits (see appendix 19 for copy of diary sheet). PEFR was recorded on a scale from 0-700 l/min and patients were instructed to take the best value of 3 blows taken sitting down prior to taking their regular morning and evening preventer inhaler. Extra pre-and post-nebuliser measurements taken by clinical teams during hospital admission were not recorded for study purposes. PEFR measurement was taken by participants using a Mini-Wright standard range EU-scale peak flow meter (Clement Clarke International Ltd, Essex, UK).

The presence or absence of a symptom in a 24 hr period was indicated by a yes or no answer. Reliever use was recorded as number of puffs of inhaler in 24 hrs or number of reliever nebulisers. The dose of any oral steroids taken on a given day was also recorded, as were days when menstruating where applicable. For Part 2, a day when a urine sample was given was also recorded.

The PEFR and symptom diaries used were prospective paper records, and while the use of prospective diary data is more accurate than retrospective recall (150), it has been observed that up to a third of patients do not measure their PEFR when asked to do so, and there is a significant drop off in recording data as time passes after contact with a clinician (151). In Part 1, 52 of 64 participants (81%) who completed the study returned their PEFR diary and 14 of 15 participants (93%) who completed Part 2 did the same. It is worth noting that many diaries were incomplete. The use of electronic devices has been shown to improve data recording and accuracy (152)

and should be considered for future studies as recommended by the ATS/ERS joint statement on asthma research (6).

2.5.8 Exacerbation

For both Parts 1 and 2 of the study, acute asthma exacerbation was defined as deterioration in symptoms severe enough to warrant treatment with oral corticosteroids, or a significant increase in dose for those already on maintenance oral steroids, either prescribed by treating clinicians, or as part of an individual patient self-management plan. Treatment dose steroids must be given for at least 3 days, consistent with the ATS/ERS definition of a severe exacerbation (6). Any treatment given by the asthma team for exacerbation was in line with pre-existing patient management plans and/or BTS guidelines.

2.5.9 Recovery

Clinical recovery was determined as patient-reported return to their pre-exacerbation state, not necessarily a return to ideal asthma control.

2.5.10 Blood and urine samples

Blood and urine samples were taken at each study visit, details of which are outlined below.

2.6 Sample collection and storage

It was planned to test blood, urine and sputum using a variety of techniques. While patient visits for both study arms would take place at hospital, Part 2 required prospective urine collection before symptoms had even developed. Initial sample collection and handling was designed to balance participants' time, volume of samples being processed, specimen degradation and detection limits of planned tests. Furthermore, samples were processed at both QAH and The University of Portsmouth (UoP). This section outlines the initial sample collection and handling.

2.6.1 Blood

Blood was taken from participants at every study visit into Vacutainer (BD UK Ltd, Oxford, UK) tubes. Samples were drawn into 1 citrate, 1 ethylenediaminetetraacetic acid (EDTA) and 1 serum separating (clot-activator) tube and sent to the PHT laboratory for full blood count (FBC), urea and electrolyte (U+E), liver function (LFT), glucose, C-reactive protein (CRP), calcium, D-dimer, fibrinogen, international normalised ratio (INR) and activated partial thromboplastin ratio (APTR). These tests were performed by the pathology services at PHT and BHNFT using commercial analysers and standard protocols. Details of the analysers in use during the study period are outlined in appendices 20 (PHT) and 21 (BNHFT). The methods employed at the two laboratories were deemed comparable prior to study commencement with the exception of IgE measurement. All IgE testing was performed at PHT for this reason.

In usual clinical practice at QAH, the D-dimer test requires a special request to limit unnecessary testing and costs. Despite samples being labelled as research specimens and prior arrangement with the laboratory being made, several samples were not processed and therefore data was missing. A more failsafe system should be considered for future projects.

Further blood was collected in EDTA tubes for plasma and processed at QAH as follows. Samples were centrifuged at 1559 g for 10 minutes in line with QAH laboratory practice. Plasma was then aspirated from the samples leaving cell layers undisturbed. If more than 1 tube was collected, plasma was mixed before aliquots were frozen and stored at -80^{o} C. It is worthy of note that although our method of plasma processing should result in plasma with a low platelet count, it was not "platelet poor plasma" recommended by the manufacturer of the Enzyme-Linked Immunosorbent Assay (ELISA) kits used. This would require a further centrifugation step, and platelet free plasma a further high speed centrifugation (153). When comparing the method used to prepare plasma with other published work that have used the same ELISA kits (154) the levels of PF4 in the present study are of a

magnitude 10x greater. The normal values reported by Peterson and colleagues (154) refer to platelet poor plasma. The different plasma preparation used in the present study would be expected to give higher platelet counts and hence would explain the higher values. The use of EDTA compared to citrate should result in less overall platelet activation (155) and hence α granule release, including PF4. Tutluoglu and colleagues (136) observed higher PF4 levels in plasma in subjects with asthma compared with healthy controls, a factor that could account for increased PF4 levels in the present study population, however, it is likely that the increased platelet count overall is responsible for this observed difference in PF4.

Plasma was subsequently analysed for PAI-1, TGF-β1, VEGF, TAFI, TF and PF4 using enzyme-linked immunosorbent assays (ELISA), and IL-5, IL-6, IL-8, IL-12P40, IL-13, IL17A, eotaxin, RANTES, interferon (IFN)-γ and TNF-α using immunofluorescence. Each assay is described below.

2.6.2 Urine

The specific requirements for each test are outlined below but in brief, a mixture of immediately frozen and "fresh" (refrigerated up to 1 week but not frozen) urine was required. The workflow for urine processed at study visits is outlined in figure 6.

Creatinine was tested on all urine samples to enable standardisation of urine sample concentrations at PHT and BNHFT laboratories. The commercial analysers used are detailed in appendices 20 and 21.

The FDP analysis required "fresh" urine that had not been frozen and defrosted. The manufacturer of this test advised that it could be performed on urine for up to a week that had been refrigerated at 4^{o}C, but degradation of the FDPs occurred with freezing, if not refrigerated, or if analysed beyond 1 week. A further planned mass spectrometry analysis required immediate processing and snap-freezing to

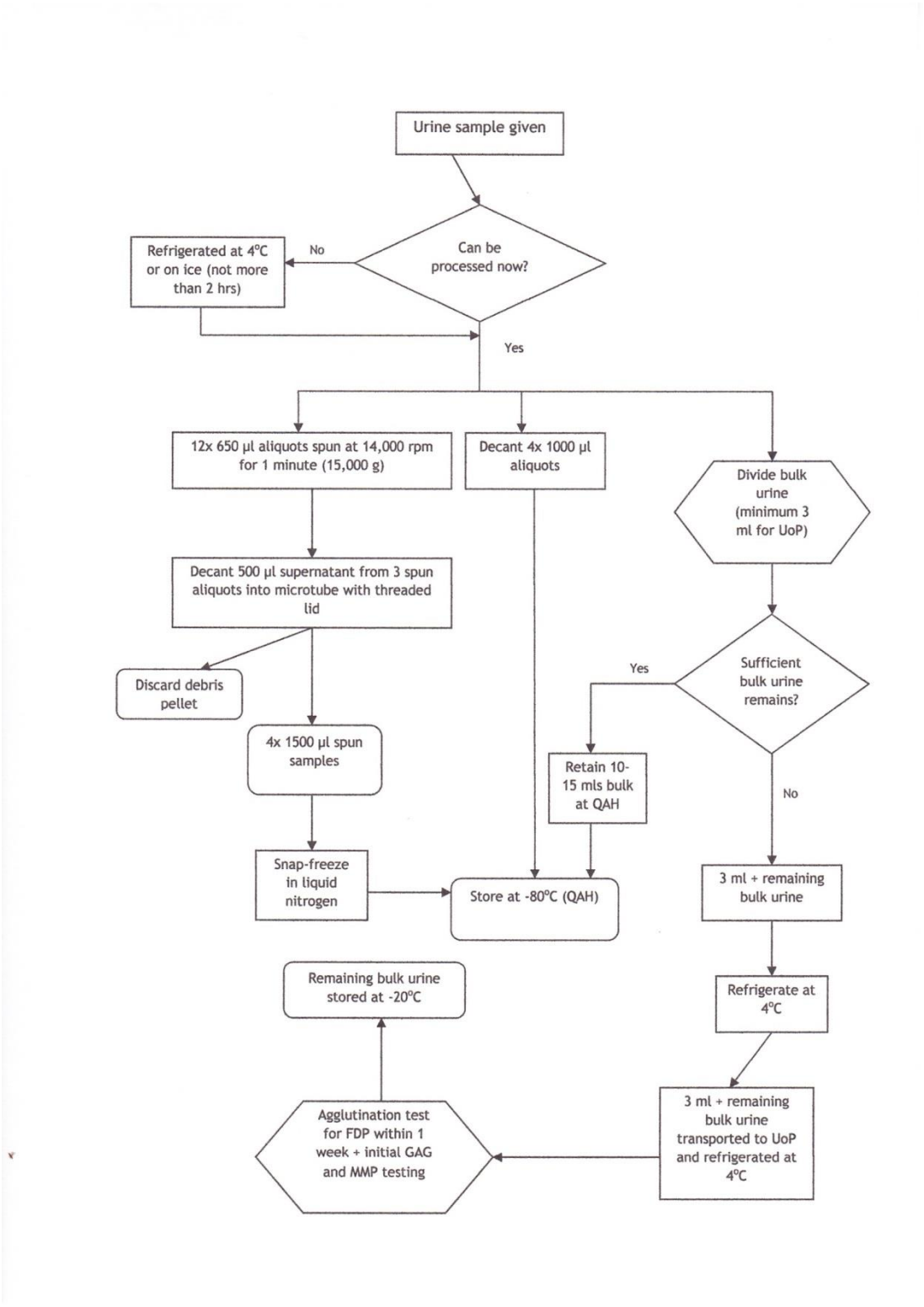
prevent protein degradation. Furthermore, sample analysis took place both at QAH and UoP laboratories.

For prospective urine samples collected in Part 2, participants were asked to give samples at home into universal specimen pots provided. Instructions were given to provide 3 samples a week. Samples should ideally be the first sample after waking up, and subsequent urination from that day or following days, should not be added to increase volume. Once given, samples should be stored at $4^{o}C$ in participants' refrigerators. Two samples should be given per week on alternate days. A weekly collection or drop off of samples was arranged, the third sample of the week was on the day of collection/drop off to maintain the "freshest" possible specimens.

Once non-visit specimens arrived at QAH, they were divided into 2x 1000 µl aliquots and 1x 15 ml bulk specimen for retention at QAH, and then the remainder was used at UoP. The processing at UoP required a minimum of 3 mls, so QAH bulk specimen volumes were adjusted to meet this requirement. Both aliquots and bulk specimens retained at QAH were frozen and stored at $-80^{o}C$. Remaining specimens for UoP were refrigerated at $4^{o}C$ and subsequently transferred to the University where they were again refrigerated until processing took place. Processing at UoP occurred within 1 week of each sample being given. After UoP processing, any remaining samples were frozen in bulk and stored at $-20^{o}C$ at UoP.

The need to process fresh urine required staff to be available at both PHT and UoP on most days. During the period from November 2011 to January 2012, one member of the research team at UoP who was performing the majority of the urine testing for FDPs, MMPs and GAGs, took a period of leave. After discussion within the research team and an unplanned statistical analysis of FDP results until that time, the decision was taken to suspend analysis of fresh urine during this period, hence missing data for these tests during this period.

Figure 6 – Urine sample handling



2.6.3 Sputum

After sputum induction or expectoration, sputum was kept on ice and processed in the research laboratory at PHT immediately. The mucoid portion of sample was selected to avoid salivary contamination and then diluted 1:9 with 10% sputolysin (Calbiochem, UK), vortexed for 10 seconds, and placed on a roller for 30 minutes. After mixing, the sample was filtered through a 100 µm cell strainer, then centrifuged at 790 g at 4^{o}C for 10 minutes. Supernatant was then removed and frozen at -80^{o}C for subsequent analysis.

The cell pellet was then re-suspended in 1 ml of PBS. 20 µl of suspension was added to 20 µl of trypan blue. Using a Cellometer vision fluorescent cell counter and Peqlab software (Peqlab Ltd, Sarisbury Green, UK), the volume required to adjust the concentration of cell suspension to 0.5x10^{6}/ml was calculated. The volume was adjusted as calculated and a cytospin was performed at 450 g for 6 minutes to produce slides. Once dry, slides were stained with eosin and methylene blue, differential cell counts were then performed manually.

Whether spontaneously expectorated or induced, sputum samples were processed in line with established protocols using the approach of selecting viscid portions of sputum (156). Expectorated and induced sputum samples have been shown to be comparable in terms of differential cell counts (147). Although supernatant from samples was frozen and stored, no further analysis was performed due to the overall paucity of samples.

2.7 Blood and urine analyses

2.7.1 Plasminogen activator inhibitor-1 (PAI-1) plasma ELISA

PAI-1 in plasma was analysed using a human PAI-1 ELISA Duoset® ELISA development system (R&D Systems, USA). A Nunc-Immuno™ 96 well MaxiSorp™ high protein-binding affinity plate (Thermo-Fisher Scientific, USA) was coated with 100 µl per well of capture antibody (mouse anti-human PAI-1 antibody) at a

working concentration of 4 µg/ml in 1x phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+}. Following incubation overnight at room temperature, the plate was washed with 200 µl per well of wash buffer (0.05% Tween 20 (Fisher Scientific, UK) in 1x PBS without Ca^{2+} or Mg^{2+}) repeated 3 times to complete the wash step. The plate was then blocked with 200 µl per well of block buffer (1% bovine serum albumin (BSA) in PBS without Ca^{2+} or Mg^{2+}) and incubated for a minimum of 1 hour at room temperature.

Samples were defrosted on ice and then diluted 1:10 with reagent diluent (1% BSA in PBS without Ca^{2+} or Mg^{2+}), and recombinant human PAI-1 was serially diluted in reagent diluent to provide a 7 point standard curve (20,000 pg/ml, 10,000 pg/ml, 5000 pg/ml, 2500 pg/ml, 1250 pg/ml, 625 pg/ml, 312.5 pg/ml) and reagent diluent as zero. After incubation with block buffer, the wash step was repeated and 100 µl of standards or samples were added to each well, in duplicate, and incubated for 2 hours at room temperature. After 2 hours incubation, the wash step was repeated and 100 µl of detection antibody (biotinylated goat anti-human PAI-1 antibody) at a working concentration of 400 ng/ml in reagent diluent with 2% normal goat serum was added to each well.

After a further 2 hours incubation at room temperature, the plate was again washed as described above and to each well was added 100 µl of streptavidin horseradish peroxidase (HRP) at a dilution of 1:200 in reagent diluent. The plate was incubated for 20 minutes at room temperature obscured from direct light. A final wash step was then performed, after which 100 µl of substrate buffer solution (tetramethylbenzidine (TMB) see appendix 22) was added to each well for a final 20 minute incubation obscured from light before 50 µl of stop solution (2 M H_2SO_4) was added per well.

The optical densities of the plate were immediately read at 450 nm with a 570 nm wavelength correction using a Dynex MRXe plate reader and Revelation version

4.25 software (Dynex technologies, USA). See appendix 23 for sample standard curve.

2.7.2 Transforming growth factor-β1 (TGF-β1) plasma ELISA

TGF-β1 in plasma was analysed using a human TGF-β1 ELISA Duoset® ELISA development system (R&D Systems, USA). A Nunc-Immuno™ 96 well MaxiSorp™ high protein-binding affinity plate (Thermo-Fisher Scientific, USA) was coated with 100 µl per well of capture antibody (mouse anti-human TGF-β1) at a working concentration of 2 µg/ml in 1x PBS without Ca^{2+} or Mg^{2+}. Following incubation overnight at room temperature, the plate was washed with 200 µl per well of wash buffer (0.05% Tween 20 (Fisher Scientific, UK) in 1x PBS without Ca^{2+} or Mg^{2+}) repeated 3 times to complete the wash step. The plate was then blocked with 200 µl per well of block buffer (5% Tween 20 (Fisher Scientific, UK) in PBS without Ca^{2+} or Mg^{2+}) and incubated for a minimum of 1 hour at room temperature.

This ELISA kit measures only active TGF-β1 therefore latent TGF-β1 must be activated by the addition of 1 M HCl. An initial test plate was run with samples that did not go undergo the activation step at a 1:2 dilution compared with the same samples after activation at a 1:6 dilution. This confirmed a significant difference following activation (<20 pg/ml vs >300 pg/ml) and all subsequent analyses included this activation step.

Plasma samples were defrosted on ice. To 40 µl of sample was added 20 µl of 1 M HCl to activate the latent TGF-β1 present. After 10 minutes incubation at room temperature, the reaction was stopped by adding 20 µl of 1.2 M NaOH/0.5 M HEPES. This activated sample was further diluted by adding 160 µl of reagent diluent (1% BSA in 0.05% Tween 20 (Fisher Scientific, UK) in 1x PBS without Ca^{2+} or Mg^{2+}), giving a final 1:6 dilution. Recombinant human TGF-β1 was serially diluted in reagent diluent to give a 7 point standard curve (2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml) and neat reagent diluent was used as a control.

After incubation with block buffer, the plate was washed as described above and to each well was added 100 µl of sample or standard in duplicate. After 2 hours incubation the plate was again washed and to each well was added 100 µl of detection antibody (biotinylated chicken anti-human TGF-β1) at a working concentration of 300 ng/ml diluted in reagent diluent. The plate was incubated for a further 2 hours at room temperature and then washed again, then 100 µl of streptavidin-HRP diluted 1:200 in reagent diluent was added to each well and incubated for 20 minutes at room temperature obscured from light. A final wash step was then performed and 100 µl of TMB substrate was added to each well and incubated obscured from light for 20 minutes before the reaction was stopped by adding 50 µl of 2 M H_2SO_4.

The plate was read immediately at 450 nm wavelength with a 570 nm wavelength correction using the same Dynex plate reader. See appendix 23 for sample standard curve.

2.7.3 Vascular endothelial growth factor (VEGF) plasma ELISA

VEGF in plasma was analysed using a human VEGF ELISA Duoset® ELISA development system (R&D Systems, USA). A Nunc-Immuno™ 96 well MaxiSorp™ high protein-binding affinity plate (Thermo-Fisher Scientific, USA) was coated with 100 µl per well of capture antibody (mouse anti-human VEGF) at a working concentration of 1 µg/ml in 1x PBS without Ca^{2+} or Mg^{2+}. Following incubation overnight at room temperature, the plate was washed with 200 µl per well of wash buffer (0.05% Tween 20 (Fisher Scientific, UK) in 1x PBS without Ca^{2+} or Mg^{2+}) repeated 3 times to complete the wash step. The plate was then blocked with 200 µl per well of block buffer (10% fetal calf serum (FCS) in 1x PBS without Ca^{2+} or Mg^{2+} and incubated at room temperature for a minimum of 1 hour.

Plasma samples were defrosted on ice and analysed undiluted. Recombinant human VEGF was serially diluted in 10% FCS in 1x PBS without Ca^{2+} or Mg^{2+} to give a 7 point standard curve (2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml,

62.5 pg/ml, 31.25 pg/ml) and neat 10% FCS in 1x PBS without Ca^{2+} or Mg^{2+} was used as control. Once the blocking step was complete the plate was washed as described and 100 µl of sample or standard was added to each well in duplicate and incubated for 2 hours at room temperature.

Once sample incubation was complete, a further wash step was performed then 100 µl of detection antibody (biotinylated goat anti-human VEGF) at a working concentration of 100 ng/ml in reagent diluent (1% BSA in 1x PBS without Ca^{2+} or Mg^{2+}) was added to each well and incubated for 2 hours at room temperature. The plate was then washed and to each well was added 100 µl of streptavidin-HRP diluted 1:200 with reagent diluent, and incubated for 20 minutes obscured from light. A final wash step was then performed before adding 100 µl of TMB substrate to each well for a final 20 minute incubation at room temperature obscured from light. The reaction was stopped after 20 minutes by adding 50 µl of 2 M H_2SO_4.

The plate was read immediately at 450 nm wavelength with 570 nm wavelength correction using the Dynex plate reader described above.

It was anticipated, based on the findings of Lee and colleagues (74) that plasma VEGF levels would be in excess of 200 pg/ml for stable asthmatics. The initial test ELISA run showed levels at a maximum of 87 pg/ml, including samples from patients during exacerbation. To evaluate whether VEGF was being bound in the plasma, a spiking experiment was performed by adding VEGF standard to a concentration of 10,000 pg/ml. The returned concentrations were between 1400 pg/ml and 2000 pg/ml, therefore 80-86% of the added VEGF was no longer detectable, presumably due to binding within plasma.

The ELISA instructions advise reagent diluents should be developed for samples other than cell culture. A further test plate with 3 standard curves of different reagent diluents (10% fetal calf serum, 50% fetal calf serum and 1% bovine serum

albumin) was run to evaluate whether this would affect non-specific binding, with no remarkable difference. See appendix 23 for sample standard curves.

2.7.4 Thrombin-activatable fibrinolysis inhibitor (TAFI) plasma ELISA

Two different ELISA methods were used to analyse plasma for TAFI due to quantities of antibodies available.

The first employed a matched pair antibodies set, TAFI-EIA (Affinity Biologicals, Ontario, Canada). The capture antibody was diluted 1:100 in carbonate-bicarbonate buffer, pH 9.6 (Sigma capsules). 100 µl per well was added to a Nunc-Immuno^{TM} 96 well MaxiSorp^{TM} high protein-binding affinity plate (Thermo-Fisher Scientific, USA) which was incubated overnight at 4^{o}C. After incubation, the wells were emptied and 100 µl of block buffer (1% BSA in 1x PBS without Ca^{2+} or Mg^{2+}) was added to each well, then incubated for 1 hour at room temperature. The plate was then washed 3 times with 200 µl of wash buffer (0.1% Tween 20 in 1 x PBS without Ca^{2+} or Mg^{2+}). A 7 point standard curve was generated using standard reference plasma (Affinity Biologicals) (2.18 ng/ml, 4.36 ng/ml, 8.73 ng/ml, 17.45 ng/ml, 34.9 ng/ml, 69.8 ng/ml and 139.6 ng/ml), diluted in sample diluent (0.1% Tween 20 in 1x PBS without Ca^{2+} or Mg^{2+}).

100 µl of sample or standard was added to each well then incubated for 2 hours at room temperature. Samples were diluted 1:500 with reagent diluent. After 2 hours incubation, the wash step was repeated and 100 µl per well of detection antibody was applied for a further 1 hour incubation at room temperature. After incubation, 100 µl of TMB substrate solution was added to each well, then the reaction was stopped after 20 minutes with 50 µl of 2 M H_2SO_4 and the plate read immediately at 490 nm using the Dynex plate reader. See appendix 23 for sample standard curve.

The second TAFI ELISA employed was a VisuLize TAFI antigen kit (Affinity Biologicals, Ontario, Canada) which was used according to manufacturers' instructions. A six point standard curve was generated using reference plasma (69.8 ng/ml, 34.9

ng/ml, 17.45 ng/ml, 8.73 ng/ml, 4.36 ng/ml, 2.18 ng/ml). Samples were diluted 1:500 with sample diluent. A pre-coated plate was washed 3 times with 200 µl per well of wash buffer, then coated with 100 µl of sample or standard and incubated for 1 hour at room temperature. The wash step was repeated then to each well was added 100 µl of detecting antibody. After a further 30 minute incubation at room temperature, the wash step was repeated and to each well was added 100 µl of TMB substrate. After 10 minutes, the reaction was stopped by adding 100 µl per well of stop solution. The plate was then read immediately at 450 nm wavelength using the Dynex plate reader. See appendix 23 for sample standard curve.

2.7.5 Tissue Factor (TF) plasma ELISA

TF in plasma was analysed using a human TF ELISA Duoset® ELISA development system (R&D Systems, USA). A Nunc-Immuno™ 96 well MaxiSorp™ high protein-binding affinity plate (Thermo-Fisher Scientific, USA) was coated with 100 µl per well of capture antibody (mouse anti-human TF) at a working concentration of 4 µg/ml diluted in 1x PBS without Ca^{2+} or Mg^{2+}. Following incubation overnight at room temperature, the plate was washed with 200 µl per well of wash buffer (0.05% Tween 20 (Fisher Scientific, UK) in 1x PBS without Ca^{2+} or Mg^{2+}) repeated 3 times to complete the wash step.

The plate was then blocked by adding 200 µl per well of reagent diluent (1% BSA in 1x PBS without Ca^{2+} or Mg^{2+}) and incubated for 1 hour at room temperature. Samples were defrosted on ice and were analysed undiluted. Recombinant human TF was serially diluted in 1% BSA in 1x PBS without Ca^{2+} or Mg^{2+} to give a 7 point standard curve (500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, 15.63 pg/ml, 7.81 pg/ml). 100 µl of sample or standard was added to each well and incubated at room temperature for 2 hours. A further wash step was then performed and 100 µl of detection antibody (biotinylated goat anti-human TF) diluted to a working concentration of 200 ng/ml in reagent diluent was added to each well and incubated for 2 hours at room temperature.

The plate was then washed and to each well was added 100 µl of streptavidin-HRP diluted 1:200 with reagent diluent, and incubated for 20 minutes obscured from light. A final wash step was then performed before adding 100 µl of TMB substrate to each well for a final 20 minute incubation at room temperature obscured from light. The reaction was stopped after 20 minutes by adding 50 µl of 2 M H_2SO_4. See appendix 23 for sample standard curve.

A test TF plate was run with 1:10 and 1:50 sample dilutions, both returned undetectable levels of TF. A spike experiment was therefore performed where a plasma sample was spiked with TF standard to a concentration of 125 pg/ml. This spiked control sample was then diluted 1:10 and 1:50. The undiluted, un-spiked plasma returned a concentration of 5 pg/ml, the 125 pg/ml undiluted sample returned a concentration of 18.5 pg/ml, after a 1:10 dilution, 53.8 pg/ml and after 1:50 85 pg/ml. This experiment confirmed there was 86% TF binding in plasma rendering it undetectable by this ELISA. This was unexpected as manufacturer's instructions for the TF ELISA explained all TF both bound and unbound should be measured by this test. Despite attempts to contact the manufacturer, no explanation was forthcoming for this finding and further TF analyses were abandoned.

2.7.6 Platelet factor 4 (PF4) plasma ELISA

PF4 in plasma was analysed using a human PF4 ELISA Duoset® ELISA development system (R&D Systems, USA). A Nunc-Immuno™ 96 well MaxiSorp™ high protein-binding affinity plate (Thermo-Fisher Scientific, USA) was coated with 100 µl per well of capture antibody (mouse anti-human PF4) at a working concentration of 2 µg/ml diluted in 1x PBS without Ca^{2+} or Mg^{2+}. Following incubation overnight at room temperature, the plate was washed with 200 µl per well of wash buffer (0.05% Tween 20 (Fisher Scientific, UK) in 1x PBS without Ca^{2+} or Mg^{2+}) repeated 3 times to complete the wash step. The plate was then blocked with 200 µl per well of block buffer (10% fetal calf serum (FCS) in 1x PBS without Ca^{2+} or Mg^{2+} and incubated at room temperature for a minimum of 1 hour.

Plasma samples were defrosted on ice and analysed diluted with reagent diluent as described below.

Recombinant human PF4 was serially diluted in sample diluent, 10% FCS in 1x PBS without Ca^{2+} or Mg^{2+} to give a 7 point standard curve (1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, 15.63 pg/ml) and neat 10% FCS in 1x PBS without Ca^{2+} or Mg^{2+} was used as control. Once the blocking step was complete the plate was washed as described and 100 µl of sample or standard was added to each well in duplicate and incubated for 2 hours at room temperature.

Once sample incubation was complete, a further wash step was performed then 100 µl of detection antibody (biotinylated goat anti-human PF4) at a working concentration of 200 ng/ml in reagent diluent (1% BSA in 1x PBS without Ca^{2+} or Mg^{2+}) was added to each well and incubated for 2 hours at room temperature. The plate was then washed and to each well was added 100 µl of streptavidin-HRP diluted 1:200 with reagent diluent, and incubated for 20 minutes obscured from light. A final wash step was then performed before adding 100 µl of TMB substrate to each well for a final 20 minute incubation at room temperature obscured from light. The reaction was stopped after 20 minutes by adding 50 µl of 2 M H_2SO_4.

The plate was read immediately at 450 nm wavelength with 570 nm wavelength correction using the Dynex plate reader described above.

A test plate was run initially with serial dilutions of 1:2500, 1:5000, 1:10,000, 1:20,000, 1:60,000, 1:120,000 and 1:240,000 and 1:480,000. This determined that 1:5000 was an appropriate dilution to bring both exacerbation and recovery samples within the detection range of the ELISA. See appendix 23 for sample standard curve.

For the subsequent sample analyses, samples were diluted 1:5000 with reagent diluent.

2.7.7 Plasma multiplex cytokine analysis

Plasma multiplex analysis was performed for IL-5, IL-6, IL-8, IL12P40, IL-13, IL-17A, IFN-γ, TNF-α, eotaxin and RANTES using the Millipore™ Milliplex© Map Mouse cytokine/chemokine kit (Millipore corp, Missouri, USA) according to manufacturer's instructions. Mouse cytokine standard was reconstituted with 250 µl deionised water to a concentration of 10,000 pg/ml and serially diluted to 2000 pg/ml, 400 pg/ml, 80 pg/ml, 16 pg/ml and 3.2 pg/ml standards with assay buffer as control. The 96 well microtitre plate was pre-wetted by adding 200 µl wash buffer per well for 10 minutes then removed.

25 µl of sample buffer and 25 µl serum matrix was added to each well, then 25 µl or sample was added according to a plate map. To each well was then added 25 µl of premixed beads and the plate was covered and incubated for 2 hrs at room temperature. After incubation, fluid was removed from wells and 2x 200 µl washes with wash buffer were performed.

25 µl of detection antibody was then added to each well and incubated at room temperature for 1 hr. After incubation 25 µl of streptavidin-phycoerythrin was added to each well and the plate was incubated for a further 30 minutes at room temperature. All fluid was then removed and a further wash cycle was performed. 150 µl of sheath fluid was then added to each well and the plate was agitated for 5 minutes on a plate shaker to re-suspend beads.

The plate was then read immediately using a Luminex 200™ analyser (Luminex corporation, Austin, Texas, USA) according to manufacturer's instructions and recommended settings. See appendix 23 for sample standard curve.

2.7.8 Urine fibrin(ogen) degradation product (FDP) analysis

The Remel Thrombo-Wellcotest (Thermo Fisher Scientific, Oxoid, Basingstoke, UK), a commercially available latex agglutination test was used according to manufacturers' instructions to analyse FDPs in urine. The minimum detectable

concentration of FDPs by this test is 2 µg/ml. It detects human fibrinogen fragments D and E which are both fibrin and fibrinogen breakdown products.

Urine stored for up to 1 week at 4^{o}C was centrifuged at 1100 g for 10 minutes at 4^{o}C in a 15 ml Falcon tube. 1 ml of supernatant was then filtered in a 5 ml syringe using a GELMAN 30 mm diameter 0.45 µm polytetrafluoroethylene (PTFE) membrane filter (PALL Life Sciences, Southampton, UK) into a 1.5 ml eppendorf tube. 40 µl of sample and 40 µl of antibody-coated latex was mixed on a black-coloured glass plate using a plate shaker at 75 rpm. A positive result is indicated by agglutination and samples were examined at 2, 10 and 30 minute time points. If sample was positive by 10 minutes, a 1:2 serial dilution was performed and samples were re-tested as above until a negative reaction occurred. A semi-quantitative value for FDP was then reported (e.g. a 1:4 dilution indicates a minimum of 8 µg/ml FDP).

2.7.9 Urine Glycosaminoglycan (GAG) analysis

The assay will detect sulphated GAGs, but not hyaluronic acid, in both free and bound form (157).

Refrigerated samples were centrifuged for 10 minutes at 4^{o}C and 1100 g. GAGs were precipitated by adding 1 ml of supernatant to 4 mls of 100% ethanol and refrigerating at 4^{o}C overnight. After precipitation, the sample was centrifuged at 4^{o}C and 1100 g for 20 minutes.

The pellet was then re-dissolved in 0.125 ml of PBS/0.5 M NaCl (10 ml 1x PBS without Ca^{2+} or Mg^{2+} + 2.92 g NaCl) by warming at 56^{o}C in an incubator on shaking, and vortex mixing at 30 minute intervals for at least 2 hours. Samples were then centrifuged for a further 10 minutes at 4^{o}C at 1100 g. The sample was then diluted 1:10 in PBS/0.5 M NaCl (10 µl sample + 90 µl PBS/0.5 M NaCl).

7.5 µl of both the dilute and neat sample is added to each well of a microtitre plate in duplicate. 250 µl of 1,9-dimethyl-methylene blue colour reagent was then added and mixed in the wells with the samples. The colour reagent consists of 500 mls H_2O + 8 mg 1,9-dimethyl-methylene blue + 1.52 g glycine + 1.19 g NaCl, adjusted to pH 3.0 with HCl.

After mixing the samples and colour reagent with a pipette 7 times, the absorbance was read at 620 nm exactly 4 ½ minutes from adding the last of the colour reagent. The standard curve was constructed using reagent blanks and heparin in a concentration range of 0-2 µg/50 µl (0 µg/50 µl, 0.25 µg/50 µl, 0.5 µg/50 µl, 1 µg/50 µl, 1.5 µg/50 µl, 2 µg/50 µl).

2.7.10 Urine matrix metalloproteinase (MMP) analysis

The EnzChek Gelatinase/Collagenase Assay Kit (Invitrogen, Life Technologies, Paisley, UK) was used to quantitatively ascertain the levels of gelatinases present in urine, according to manufacturers' instructions.

Urine was refrigerated at 4^{o}C prior to processing then centrifuged at 4^{o}C for 10 minutes at 1100 g as universal preparation for all analyses outlined above. A further centrifugation step was performed again at 4^{o}C for 10 minutes but for 1910 g. A 96-well fluoronunc microplate (Thermo-Fisher Scientific, USA) was used. To each well in duplicate was added 100 µl of standard, sample or control and 80 µl of 1x reaction buffer. Next to these wells in duplicate was added 100 µl of standard, sample or control with 60 µl of 1x reaction buffer and 20 µl of 10 mM 1,10-phenanthroline. Control was 1x reaction buffer and standards 0.001, 0.01 and 0.1U/ml of collagenase (from Clostridium histolyticum). Samples were undiluted and taken from supernatant after centrifugation taking care not to disturb any pellet formed.

Plates were kept on ice and to each well was added 20 µl of 100 µg/ml DQ gelatin giving a total volume of 200 µl per well. Plates were read immediately using a

fluorescence microplate reader at 495 nm and fluorescence emission at 515 nm. Each plate run was for 2 hours and sequential readings were taken at time 0 and every 10 minutes for the duration of the run.

3.0 PART 1 RESULTS

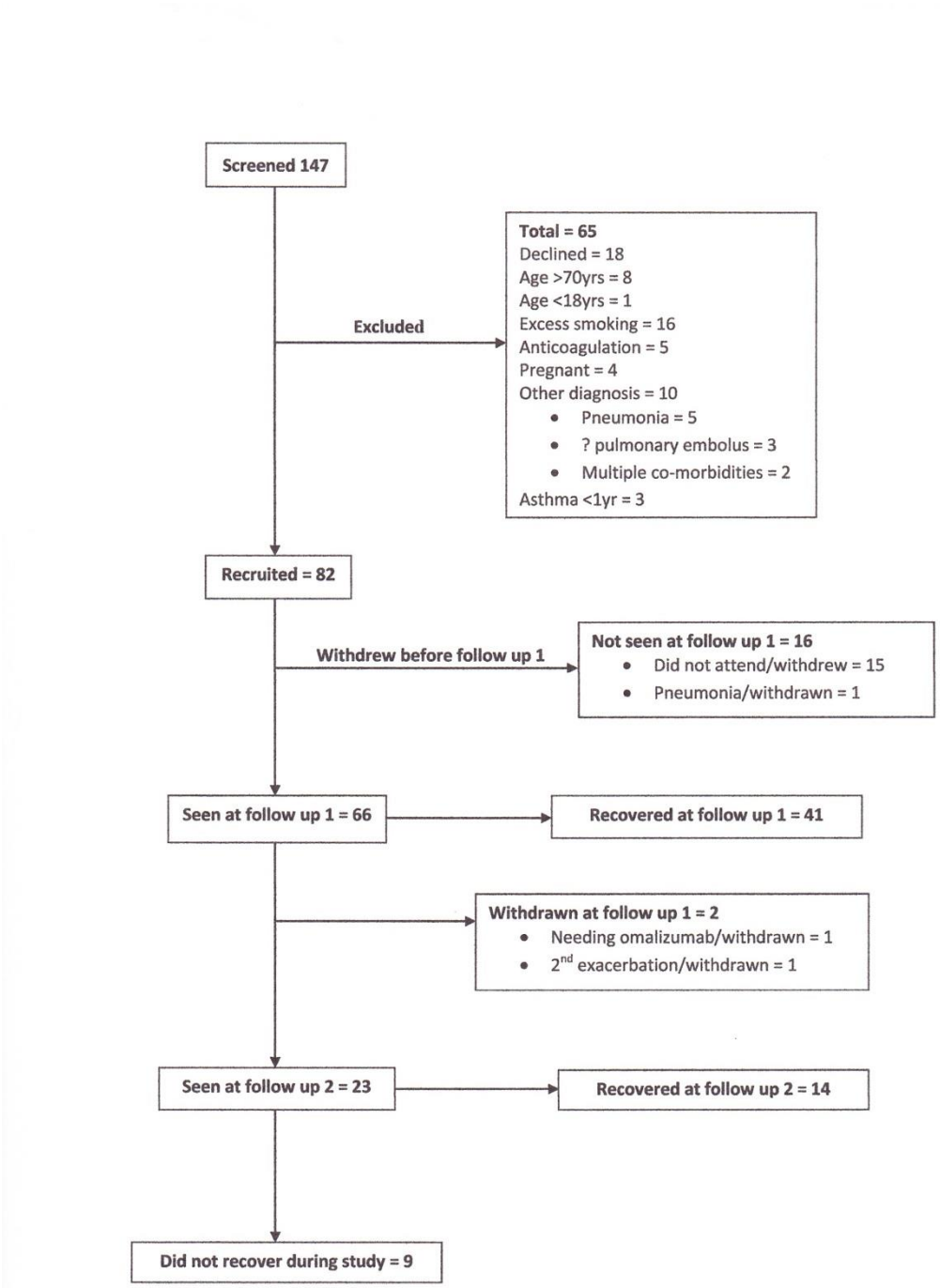
3.1 Part 1 screening, recruitment, withdrawal and completion

We successfully recruited 82 patients with acute exacerbation of asthma, 64 of whom completed the study. This is the largest study to date investigating coagulation and fibrinolysis in acute exacerbation of asthma.

A total of 147 patients were screened for Part 1 and 82 participants were recruited to this arm of the study. The reasons for study exclusion at screening are outlined below in figure 7. Of these 82, 16 were not seen at follow up, 15 of which did not attend and hence withdrew, and 1 was diagnosed with pneumonia the day after study recruitment and hence withdrawn. The remaining 66 were seen at follow up visit 1 and 2 were withdrawn at this time, one because they experienced a second exacerbation and the other required omalizumab treatment at review necessitating withdrawal.

Of the remaining 64 participants, 41 recovered at follow up 1 and the remaining 23 were offered a second follow up appointment. All 23 attended their second follow up and 14 had recovered at this visit, leaving 9 study participants who did not recover during the study period. See figure 7 below for summary of Part 1 recruitment.

Figure 7 – Part 1 screening



3.1.1 Adverse events – Part 1

There were 7 adverse events during the study period (table 4), all classified as serious adverse events (SAE), 6 due to participants being admitted to hospital, and 1 because the participant had a prolonged hospital stay.

Table 2 – Adverse events – Part 1

Study number*	Adverse event	Related to study activity?
J1013XX	Re-admission to hospital with asthma	No
J1015XX	Re-admission to hospital with asthma	No
J1027XX	Pneumonia after initial admission	No
J1030XX	Re-admission to hospital with asthma	No
J1042XX	Re-admission to hospital with asthma	No
J1052XX	Re-admission to hospital with asthma	No
J1069XX	Admitted for observation after induced sputum	Yes

* Participant initials removed to preserve anonymity

Only 1 SAE was deemed to be related to study activity, but was subsequently deemed by the study sponsor a recognised complication of the procedure (sputum induction) that was addressed in the study protocol and design.

3.2 Participant characteristics – Part 1

Patient characteristics were summarised for all patients in Part 1 of the study. Continuous variables were summarised by either the mean and standard deviation, if found to be normally distributed, or median and inter-quartile range if not normally distributed. Categorical variables were summarised by the number and percentage of patients in each category.

Comparisons were made between patients who did and did not complete the study. The analysis of continuous variables found to be normally distributed was performed using the unpaired t-test, whilst continuous variables not found to be normally distributed were analysed using the Mann-Whitney test. Binary categorical variables, and those with no ordering of the categories were analysed

using Fisher's exact test. Ordinal categorical variables were analysed using the Mann-Whitney test (in order to take account of the order of the categories, which is not utilised using Fisher's exact test).

Results

A summary of the patient characteristics is given in the two subsequent tables (table 3 continuous variables and table 4 categorical). The figures reported are the results for all patients, and then split by those who withdrew from the study and those who did not. P-values indicating the significance of the difference between those who did and did not withdraw are also presented.

Table 3 – Participant characteristics Part 1 continuous variables

Variable	All patients (n=82)	Completed (n=64)	Withdrawn (n=16)	P-value
Age (years) (*)	40.1 (13.4)	42.1 (13.5)	32.8 (10.2)	**0.008**
Hospital admissions (**)	0 (0, 2)	0 (0, 1)	1 (0, 2)	**0.02**
Steroid courses (**)	3 (1, 6)	2 (1, 6)	4 (2, 9)	0.13
Body mass index (BMI) (kg/m^2) (**)	28.9 (25.0, 34.8)	30.5 (25.4, 35.6)	27.7 (24.7, 31.2)	0.14

(*) Mean (standard deviation) reported

(**) Median (inter-quartile range) reported

The results suggested a significant difference in age and number of hospital admissions due to asthma between those who withdrew and those who completed the study. Those who withdrew tended to be younger, with a mean age of 32, compared to 42.

No significant differences were observed for the number of steroid courses over 2 years or for BMI comparing study subjects who withdrew and those who completed the study.

Table 4 – Participant characteristics Part 1 categorical variables					
Variable	Category	All patients (n=82)	Completed (n=64)	Withdrawn (n=18)	P-value
Sex	Male	26 (32%)	22 (34%)	4 (22%)	0.40
	Female	56 (68%)	42 (66%)	14 (78%)	
GINA 2008	Intermittent	8 (10%)	7 (11%)	1 (6%)	0.07
	Mild Persist.	20 (24%)	17 (27%)	3 (17%)	
	Mod. Persist	25 (30%)	21 (33%)	4 (22%)	
	Severe Persist	29 (35%)	19 (30%)	10 (56%)	
Asthma Severity	Mild	27 (33%)	22 (34%)	5 (28%)	0.55
	Moderate	23 (28%)	18 (28%)	5 (28%)	
	Severe	32 (39%)	24 (38%)	8 (44%)	
Eosinophilic	No	33 (40%)	27 (42%)	6 (33%)	0.59
	Yes	49 (60%)	37 (58%)	12 (67%)	
Smoking status	Current	14 (17%)	10 (16%)	4 (22%)	0.14
	Ex	24 (29%)	16 (25%)	8 (44%)	
	Never	44 (54%)	38 (59%)	6 (33%)	
Obese	No	40 (50%)	28 (44%)	12 (71%)	0.10
	Yes	40 (50%)	35 (56%)	5 (29%)	
Atopy (SPT positive)	No	13 (22%)	13 (22%)	0 (0%)	1.00
	Yes	47 (78%)	46 (78%)	1 (100%)	
Atopy (raised IgE)	No	30 (38%)	25 (40%)	5 (31%)	0.58
	Yes	49 (62%)	38 (60%)	11 (69%)	

Comparing categorical patient characteristics, there was no significant difference between those who completed and those who withdrew from the study. Participants who withdrew tended towards more severe asthma as defined by GINA 2008 criteria, although this result was only of borderline statistical significance.

Discussion

Study recruitment into Part 1 of the study took place in 2 centres, BNHFT Basingstoke and QAH Portsmouth, both of which are located in Hampshire, UK. The population served by both centres is predominantly white Caucasian, and although not formally recorded in the CRF data, this was reflected in the study population recruited. The majority of the study recruits were female (68%), consistent with the

known trends in asthma prevalence among adults (158). In terms of asthma inflammatory phenotype, our study population was predominantly eosinophilic (60%) and/or atopic (78% by skin prick test positivity, 62% by elevated IgE levels). This is consistent with the observation that eosinophilia is most commonly seen in classic atopic asthma (159), however, the definition of eosinophilic asthma and variability in the presence of eosinophilia must be considered and is discussed below alongside the definitions used in this study.

Comparing participants who completed the study compared with those who withdrew, those who withdrew were on average 10 years younger (p=0.008) and were more likely to have had an exacerbation requiring hospital admission within the 2 years preceding study enrolment (p=0.02). There were no other significant differences in terms of patient demographics between those who withdrew and those who completed the study. Admission to hospital within the previous year is recognised as a risk factor for death from asthma (4, 158). Furthermore, failure to attend appointments is also recognised as a risk for asthma death (4). It is worth noting that the reason for withdrawal from Part 1 of the study in 15 of 18 patients (83%) was failure to attend follow up. Other risks for asthma death such as adverse psychosocial factors (4, 158) were not recorded as part of the data collected for this study but should be considered in the design of future projects. Our study recorded hospital admission for asthma in the preceding 2 years, whereas 1 year would be consistent with evidence of risk for asthma death and of greater relevance. The significance of the younger age of those who withdrew is not clear from the data collected and potential factors such as employment, family or financial commitments could just as easily explain a younger or older age bias so do not narrow speculation on this matter.

Overall it is worth taking the above factors into consideration if extrapolating the findings of this study when looking at different populations.

3.3 Plasma and urine analyses – Part 1

Plasma and urine markers of fibrin turnover and cytokines were measured at admission, and then at two subsequent follow-up timepoints. The admission visit in Part 1 corresponds with asthma exacerbation and subsequent follow up visits are after treatment with oral corticosteroids and a period of recovery from exacerbation. To simplify the analyses comparisons were performed between values at admission (exacerbation) and values at each of two follow-up timepoints separately.

The majority of variables were measured on a continuous scale. The statistical methods used were dependent on the distribution of the values. Where the values were normally distributed, the paired t-test was used to compare between timepoints. For a large number of the variables the values were not normally distributed, and the Wilcoxon matched-pairs test was preferred.

One variable was measured on a binary scale. The paired exact test was used to compare this variable between timepoints.

Results

The first set of analyses examined the change in values from admission to the first follow-up timepoint, and a summary of the results is given in the table 5 below. The first figures reported are the number of patients with measurements at both timepoints. For those variables where the values were not normally distributed, the median and inter-quartile range at each timepoint is reported, along with the median difference and corresponding confidence interval. For those variables where the values were normally distributed, the mean and standard deviation at each timepoint is reported, along with the mean difference and corresponding confidence interval. P-values indicating the significance of the results are also given.

Table 5 – Part 1 plasma and urine results admission vs follow up 1					
Variable	N	Admission median (IQR) or mean (SD)(*) or number (%)(**)	Follow-up 1 median (IQR) or mean (SD)(*) or number (%)(**)	Change exacerbation - follow up 1 median (95% CI)	P-value
Plasma Fibrin markers					
TAFI (ng/ml)(*) (†)	59	6360 (2573)	7306 (2739)	945 (13, 1878)	0.05
PAI-1 (pg/ml)	59	15400 (10400, 29300)	16500 (9273, 34900)	1700 (-1704, 3868)	0.55
D-dimer (µgFEU/ml)	59	0.19 (0.13, 0.37)	0.21 (0.15, 0.28)	0.0 (-0.07, 0.04)	0.40
Fibrinogen (g/l) (*)	59	3.00 (0.85)	2.92 (0.61)	-0.08 (-0.30, 0.13)	0.45
Plats (10^*9/l) (*)	32	276 (88)	278 (77)	2 (-18, 22)	0.82
PF4 (µg/ml)(*)	56	1.8 (1.6)	2.3 (1.9)	0.6 (0.1, 1.1)	**0.03**
TGFβ1 (pg/ml)	59	1637 (759, 3138)	1619 (1020, 2534)	63 (-192, 344)	0.78
VEGF (pg/ml)	59	2.6 (0.0, 28.2)	5.3 (0.0, 37.0)	0.0 (0.0, 0.0)	0.92
Eosinophils (10^*9/l)	32	0.10 (0.00, 0.48)	0.30 (0.20, 0.48)	0.13 (0.10, 0.20)	**0.003**
Plasma Cytokines					
IL-6 (pg/ml)	57	1.4 (0.0, 5.1)	0.9 (0.0, 2.3)	0.0 (-1.4, 0.0)	**0.02**
IFN (pg/ml)	57	24 (8, 49)	19 (8, 32)	-3 (-10, 0)	**0.003**
IL-13 (pg/ml)	57	0.0 (0.0, 4.5)	0.0 (0.0, 2.0)	0.0 (-0.2, 0.0)	**<0.001**
RANTES (pg/ml)(*)	57	2736 (1193)	2134 (873)	-601 (-938, -266)	**<0.001**
IL-12 P40 (pg/ml)	57	16 (0, 49)	7 (0, 39)	0 (-30, 2)	**0.01**
IL-17A (pg/ml)	57	3.4 (0.0, 13.1)	1.9 (0.0, 6.4)	-0.3 (-3.2, 0.0)	**0.002**
IL-8 (pg/ml)	57	2.6 (1.2, 5.9)	2.5 (1.7, 4.4)	0.2 (-0.7, 0.7)	0.56
TNF-α (pg/ml)	57	7.2 (4.5, 11.0)	6.6 (4.6, 10.0)	-0.9 (-1.6, 0.6)	0.11
Eotaxin (pg/ml)	57	53 (42, 69)	63 (49, 80)	9 (2, 17)	**0.002**
IL-5 (pg/ml)	57	3.0 (1.1, 7.0)	1.9 (1.2, 4.6)	-0.4 (-1.5, 0.0)	**0.007**
Urine tests					
FDP (+/-)(**)	47	10 (21%)	8 (17%)	-4% (-23%, 15%)	0.80
MMP activity (FU)	5	0.0014 (0.0009,0.0026)	0.0008 (0.003, 0.0023)	-0.0006 (-0.008, 0.0005)	0.14
GAG:Creatinine (µg/mmol)	27	0.44 (0.29, 0.62)	0.29 (0.19, 0.40)	-0.21 (-0.27, 0.03)	**0.04**

(*) Mean (standard deviation) reported, along with mean change (95% CI)

(**) Number (%) with positive responses reported, along with percentage change (95% CI)

(†) Omitting one patient with extremely high value at follow-up

The results suggested a number of significant changes between admission and follow-up 1.

There were significantly higher values in plasma samples taken at follow up 1 compared with admission for PF4, eosinophils and eotaxin. For example, there was a median increase in eosinophils of 0.13 x10*9/l. There was also slight evidence of an increase in TAFI, but the result for this variable was only of borderline statistical significance.

Conversely there was a significant decrease from admission to follow up 1 for GAGs, IL-6, IFN, IL-13, RANTES, IL-12, IL-17A and IL-5. For example, the mean decrease in RANTES was 601 pg/ml between the two study visits. There was a median reduction of 0.21 µg/mmol over time for GAGs.

A similar set of analyses were performed to examine the change in the various parameters from admission to the second follow-up visit. The results are summarised in the table 6 below.

Table 6 – Part 1 plasma and urine admission vs follow up 2					
Variable	N	Admission median (IQR) or mean (SD)(*) or number (%)(**)	Follow-up 2 median (IQR) or mean (SD)(*) or number (%)(**)	Change admission – follow up 2 median (95% CI)	P-value
Plasma Fibrin markers					
TAFI (ng/ml)(*)	21	5956 (1776)	7242 (2915)	1285 (-151, 2722)	0.08
PAI-1 (pg/ml)	21	14200 (6811, 23300)	17400 (7301, 28650)	6200 (-2191, 10240)	**0.04**
D-dimer (µgFEU/ml)	22	0.19 (0.10, 0.34)	0.26 (0.14, 0.33)	0.03 (-0.06, 0.10)	0.53
Fibrinogen (g/l)(*)	22	2.97 (0.99)	3.12 (0.70)	0.15 (-0.22, 0.51)	0.41
Plats (10^*9/l)(*)	9	241 (71)	270 (82)	30 (1, 58)	0.05
PF4 (µg/ml)(*)	21	1.8 (1.4)	1.9 (1.5)	0.1 (-0.7, 1.0)	0.80
TGFβ1 (pg/ml)	21	1793 (874, 3652)	1332 (648, 2334)	-351 (-1625, 114)	0.10
VEGF (pg/ml)	21	0.3 (0.0, 31.6)	7.2 (0.0, 35.0)	0.0 (0.0, 3.8)	0.68
Eosinophils (10^*9/l)	9	0.00 (0.00, 0.13)	0.20 (0.10, 0.70)	0.10 (0.00, 0.80)	**0.04**
Plasma Cytokines					
IL-6 (pg/ml)	20	0.8 (0.2, 5.1)	1.9 (0.0, 4.7)	0.1 (-0.3, 1.9)	0.60
IFN (pg/ml)	20	18 (8, 39)	19 (10, 39)	1 (-21, 20)	0.82
IL-13 (pg/ml)	20	0.0 (0.0, 6.9)	0.0 (0.0, 12.4)	0.0 (0.0, 1.0)	0.83
RANTES (pg/ml)(*)	20	2568 (1128)	2374 (1257)	-193 (-785, 398)	0.50
IL-12 P40 (pg/ml)	20	18 (0, 46)	3 (0, 52)	0 (-4, 26)	0.68
IL-17A (pg/ml)	20	1.4 (0.0, 11.7)	2.5 (0.0, 15.3)	0.1 (-0.2, 5.4)	0.41
IL-8 (pg/ml)	20	2.6 (1.2, 7.8)	3.3 (2.2, 6.2)	0.4 (-2.3, 1.3)	0.85
TNF-α (pg/ml)	20	7.3 (4.5, 11.5)	8.1 (5.8, 13.2)	1.5 (0.0, 4.9)	0.35
Eotaxin (pg/ml)	20	57 (43, 71)	63 (51, 90)	12 (-5, 23)	0.07
IL-5 (pg/ml)	20	2.9 (1.0, 5.9)	2.4 (1.3, 7.9)	0.0 (-1.1, 2.4)	0.99
Urine tests					
FDP (+/-)(**)	17	5 (29%)	2 (12%)	-18% (-48%, 13%)	0.38
MMP activity (FU)	3	0.0015 (0.0009, 0.0018)	0.0005 (0.0002, 0.0014)	-0.0004 (-0.0016, 0.0000)	0.11
GAG:creatinine (µg/mmol)	4	0.37 (0.16, 0.51)	0.47 (0.13, 1.24)	0.26 (-0.49, 1.46)	0.47

(*) Mean (standard deviation) reported, along with mean change (95% CI)

(**) Number (%) with positive responses reported, along with percentage change (95% CI)

The results suggested few significant differences between admission and follow-up 2.

There was a significant increase in PAI-1 from admission to follow up 2, with a median increase of 6200 pg/ml. There was also a significant increase in eosinophils, with a median increase of 0.10 x10*9/l.
Additionally there was some evidence of an increase in TAFI, platelets and eotaxin at follow up 2 compared with admission, but these results were not quite statistically significant.

It should be noted that although there were fewer differences from admission to the second follow-up timepoint, this may be partly attributable to smaller numbers of patients with measurements at this latter follow-up point. Some of the lack of significance may be due to smaller differences, but some may be due to lower power with smaller numbers. For example, the change in eotaxin from admission to follow-up 2 was greater than from admission to follow-up 1, but the result was less statistically significant due to the smaller numbers.

3.3.1 Exacerbation vs clinical recovery – Part 1

Although the study incorporated 2 follow up timepoints, those participants who recovered at their first follow up visit did not require a second follow up, and conversely, only those who had not clinically recovered at follow up 1 were offered a second follow up visit. Furthermore, some participants did not clinically recover at second follow up.

From a clinical perspective, the comparison between exacerbation and clinical recovery is perhaps more pertinent than a 35 day or 65 day temporal interval.

Data from participants who clinically recovered at the first follow up at 35 days (rapid recovery), were grouped with data from the second follow-up timepoint at

65 days (delayed recovery), and compared with admission visit data. Patients who did not recover were omitted from the analyses.

The majority of variables were measured on a continuous scale. The statistical methods used were dependent on the distribution of the values. Where the values were normally distributed, the paired t-test was used to compare between timepoints. For a large number of the variables the values were not normally distributed, and the Wilcoxon matched-pairs test was preferred.

One variable was measured on a binary scale. The paired exact test was used to compare this variable between timepoints.

Results – exacerbation vs clinical recovery Part 1

The analyses examined the change in values from exacerbation to recovery and a summary of the results is given in table 7 below. The first figures reported are the number of patients with measurements at both timepoints. For those variables where the values were not normally distributed, the median and inter-quartile range at each timepoint is reported, along with the median difference and corresponding confidence interval. For those variables where values were normally distributed, the mean and standard deviation at each timepoint is reported, along with the mean difference and corresponding confidence interval. P-values indicating the significance of the results are also given.

Table 7 – Part 1 plasma and urine exacerbation vs clinical recovery					
Variable	N	Exacerbation median (IQR) or mean (SD)(*) or number (%)(**)	Clinical recovery median (IQR) or mean (SD)(*) or number (%)(**)	Change exacerbation – clinical recovery median (95% CI)	P-value
Plasma fibrin markers					
TAFI (ng/ml) (*) (†)	50	6283 (2504)	7205 (2406)	921 (-39, 1882)	0.06
PAI-1 (pg/ml)	50	15900 (10550, 29325)	16550 (8089, 33175)	1219 (-1963, 4518)	0.53
D-dimer (µgFEU/ml)	48	0.21 (0.13, 0.36)	0.21 (0.14, 0.31)	0.00 (-0.07, 0.03)	0.47
Fibrinogen (g/l)(*)	48	2.89 (0.66)	2.87 (0.61)	-0.02 (-0.25, 0.22)	0.87
Plats (10^*9/l)(*)	20	282 (89)	274 (75)	-8 (-38, 22)	0.59
PF4 (µg/ml)(*)	47	1.9 (1.7)	2.2 (1.8)	0.3 (-0.4, 0.9)	0.41
TGFβ1 (pg/ml)	50	1702 (805, 2832)	1609 (921, 2411)	-63 (-331, 354)	0.92
VEGF (pg/ml)	50	6.1 (0.0, 30.9)	10.4 (0.0, 37.1)	0.0 (0.0, 0.0)	0.76
Eosinophils (10^*9/l)	20	0.00 (0.00, 0.33)	0.30 (0.20, 0.40)	0.15 (0.10, 0.29)	**0.02**
Plasma Cytokines					
IL-6 (pg/ml)	48	1.4 (0.0, 3.4)	1.0 (0.0, 4.1)	0.0 (-0.7, 0.3)	0.56
IFN (pg/ml)	48	24 (6, 50)	21 (10, 33)	-1 (-7, 2)	0.12
IL-13 (pg/ml)	48	0.0 (0.0, 4.3)	0.0 (0.0, 2.1)	0.0 (0.0, 0.0)	0.07
RANTES(pg/ml) (*)	48	2813 (1264)	2410 (1075)	-403 (-800, -6)	0.05
IL-12 P40 (pg/ml)	48	9 (0, 40)	7 (0, 50)	0 (-1, 2)	0.66
IL-17A (pg/ml)	57	3.3 (0.0, 10.5)	2.5 (0.2, 8.7)	0.0 (-1.4, 0.7)	0.42
IL-8 (pg/ml)	57	2.3 (1.2, 5.4)	2.6 (1.7, 4.5)	0.4 (-2.0, 1.2)	0.61
TNF-α (pg/ml)	57	6.3 (4.2, 10.1)	8.2 (5.6, 11.8)	0.8 (-0.8, 1.8)	0.31
Eotaxin (pg/ml)	57	51 (39, 66)	59 (49, 84)	12 (3, 22)	**<0.001**
IL-5 (pg/ml)	57	2.8 (1.1, 5.9)	2.4 (1.3, 5.5)	-0.1 (-0.9, 0.2)	0.20
Urine tests					
FDP (+/-)(**)	43	9 (21%)	9 (21%)	0% (-19%, 19%)	1.00
MMP activity (FU)	7	0.0015 (0.0009, 0.0018)	0.0008 (0.0002, 0.0020)	-0.0006 (-0.0013, 0.0003)	0.06
GAG:creatinine (µg/mmol)	17	0.44 (0.24, 0.62)	0.32 (0.16, 0.63)	-0.11 (-0.39, 0.19)	0.59

(*) Mean (standard deviation) reported, along with mean change (95% CI)

(**) Number (%) with positive responses reported, along with percentage change (95% CI)

(†) Omitting one patient with extremely high value at follow-up

The results suggested strong evidence of a change from exacerbation to recovery for only two of the parameters, eosinophils and eotaxin. Both of these parameters increased from exacerbation to recovery, the median increase in eosinophils being 0.15 x10*9/l, whilst the median increase in eotaxin was 12 pg/ml.

There was also some evidence of increase from exacerbation to recovery for TAFI, and a decrease for IL-13, RANTES and MMPs between the same visits, but these results were not quite statistically significant.

3.3.2 Rapid and delayed recovery – Part 1

As outlined above study participants in Part 1 either recovered by their first follow up visit at 35 days, by their second follow up at 65 days, or did not clinically recover during the study period. From a clinical perspective it is useful to be able to distinguish between those who rapidly recover and those who have a delayed recovery as this may impact on management of such groups. Those participants who had recovered by 35 days were classified as rapid recovery and those by 65 days a delayed recovery. The numbers of recruits who did not recover during the study period was too small to make any viable comparison.

Results – exacerbation vs rapid recovery Part 1

Analyses were performed to examine the change in values from exacerbation to first follow-up in patients who recovered rapidly. A summary of the results is given in table 8 below. The first figures reported are the number of patients with measurements at both timepoints. For those variables where the values were not normally distributed, the median and inter-quartile range at each timepoint is reported, along with the median difference and corresponding confidence interval. For those variables where the values were normally distributed, the mean and standard deviation at each timepoint is reported, along with the mean difference and corresponding confidence interval. P-values indicating the significance of the results are also given.

Table 8 – Part 1 plasma and urine exacerbation vs rapid recovery					
Variable	N	Exacerbation median (IQR) or mean (SD)(*) or number (%)(**)	Rapid recovery median (IQR) or mean (SD)(*) or number (%)(**)	Change exacerbation - rapid recovery median (95% CI)	P-value
Plasma Fibrin markers					
TAFI (ng/ml)(*) (†)	36	6395 (2860)	7262 (2397)	867 (-300, 2034)	0.14
PAI-1 (pg/ml)	36	16950 (10825, 29375)	16050 (8251, 34325)	238 (-2992, 3409)	0.83
D-dimer (µgFEU/ml)	35	0.20 (0.15, 0.38)	0.20 (0.17, 0.27)	0.00 (-0.07, 0.05)	0.37
Fibrinogen (g/l)(*)	35	2.94 (0.66)	2.82 (0.63)	-0.11 (-0.37, 0.14)	0.38
Plats (10^*9/l)(*)	19	286 (89)	279 (73)	-7 (-39, 25)	0.64
PF4 (µg/ml)(*)	33	1.8 (1.8)	2.2 (1.9)	0.4 (-0.4, 1.2)	0.30
TGFβ1 (pg/ml)	36	1421 (752, 2532)	1593 (938, 2196)	112 (-187, 503)	0.36
VEGF (pg/ml)	36	5.0 (0.0, 25.9)	6.2 (0.0, 28.9)	0.0 (-1.0, 0.0)	0.94
Eosinophils (10^*9/l)	19	0.00 (0.00, 0.40)	0.30 (0.20, 0.40)	0.10 (0.07, 0.23)	**0.04**
Plasma Cytokines					
IL-6 (pg/ml)	35	1.4 (0.0, 3.4)	0.4 (0.0, 3.3)	0.0 (-1.4, 0.0)	0.17
IFN (pg/ml)	35	24 (7, 52)	20 (10, 31)	-3 (-16, 0)	**0.01**
IL-13 (pg/ml)	35	0.0 (0.0, 2.9)	0.0 (0.0, 0.3)	0.0 (-0.3, 0.0)	**0.01**
RANTES (pg/ml) (*)	35	2866 (1287)	2342 (966)	-524 (-1003, -45)	**0.03**
IL-12 P40 (pg/ml)	35	8 (0, 46)	7 (0, 42)	0 (-11, 1)	0.20
IL-17A (pg/ml)	35	3.4 (0.0, 12.0)	2.4 (0.6, 7.6)	0.0 (-4.1, 0.4)	0.08
IL-8 (pg/ml)	35	2.4 (1.1, 5.1)	2.2 (1.5, 3.8)	0.2 (-0.8, 0.7)	0.45
TNF-α (pg/ml)	35	6.2 (4.2, 9.9)	7.6 (5.6, 11.6)	0.1 (-1.2, 1.3)	0.86
Eotaxin (pg/ml)	35	51 (38, 65)	58 (48, 81)	12 (3, 22)	**0.002**
IL-5 (pg/ml)	35	2.9 (1.1, 7.9)	2.2 (1.3, 5.0)	-0.4 (-2.0, 0.0)	**0.03**
Urine tests					
FDP (+/-)(**)	30	5 (17%)	7 (23%)	7% (-17%, 31%)	0.75
MMP activity (FU)	4	0.0015 (0.0010, 0.0030)	0.0014 (0.0003, 0.0025)	-0.0007 (-0.0008, 0.0004)	0.14
GAG:creatinine (µg/mmol)	15	0.44 (0.27, 0.62)	0.32 (0.17, 0.70)	-0.11 (-0.39, 0.19)	0.53

(*) Mean (standard deviation) reported, along with mean change (95% CI)

(**) Number (%) with positive responses reported, along with percentage change (95% CI)

(†) Omitting one patient with extremely high value at follow-up

The results suggested evidence of a statistically significant change from exacerbation to rapid recovery for eosinophils, IFN, IL-13, RANTES, eotaxin and IL-5. Of these variables, there was a significant increase in eosinophils and eotaxin, whilst there was a significant decrease in IFN, IL-13, RANTES and IL-5.

Results – exacerbation vs delayed recovery Part 1

The next set of analyses examined the change in values from exacerbation to 2nd follow up in patients with a delayed recovery. A summary of the results is given in table 9 below.

Table 9 – Part 1 plasma and urine exacerbation vs delayed recovery					
Variable	N	Exacerbation median (IQR) or mean (SD)(*) or number (%)(**)	Delayed recovery median (IQR) or mean (SD)(*) or number (%)(**)	Change exacerbation – delayed recovery median (95% CI)	P-value
Plasma Fibrin markers					
TAFI (ng/ml) (*)	14	5995 (1215)	7057 (2514)	1062 (-849, 2974)	0.25
PAI-1 (pg/ml)	14	15900 (9674, 27200)	17350 (7725, 32200)	6530 (-3152, 13988)	0.16
D-dimer (µgFEU/ml)	13	0.22 (0.09, 0.30)	0.25 (0.12, 0.32)	0.03 (-0.11, 0.10)	0.92
Fibrinogen (g/l)(*)	13	2.75 (0.67)	2.99 (0.56)	0.23 (-0.33, 0.80)	0.38
Plats (10^*9/l) (*)	1	*insufficient*	*data*		
PF4 (µg/ml) (*)	14	2.4 (1.3)	2.3 (1.5)	-0.1 (-1.3, 1.2)	0.91
TGFβ1 (pg/ml)	14	2564 (1226, 4019)	2057 (840, 3976)	-718 (-1958, 845)	0.30
VEGF (pg/ml)	14	16 (0, 52)	26 (0, 48)	0 (0, 9)	0.44
Eosinophils (10^*9/l)	1	*insufficient*	*data*		
Plasma Cytokines					
IL-6 (pg/ml)	13	0.8 (0.1, 5.4)	2.5 (0.0, 6.2)	0.7 (-0.6, 2.7)	0.36
IFN (pg/ml)	13	20 (3, 36)	22 (10, 42)	2 (-15, 20)	0.38
IL-13 (pg/ml)	13	0.0 (0.0, 11.4)	1.1 (0.0, 17.8)	0.0 (-3.9, 2.4)	0.72
RANTES (pg/ml) (*)	13	2671 (1237)	2593 (1354)	-78 (-854, 698)	0.83
IL-12 P40 (pg/ml)	13	14 (0, 36)	1 (0, 59)	0 (-3, 30)	0.24
IL-17A (pg/ml)	13	0.2 (0.0, 10.3)	5.6 (0.0, 16.2)	0.0 (-0.9, 8.7)	0.27
IL-8 (pg/ml)	13	2.3 (1.2, 8.8)	3.7 (2.6, 6.6)	0.6 (-3.1, 1.5)	0.92
TNF-α (pg/ml)	13	6.4 (4.3, 11.0)	9.3 (5.6, 15.2)	2.4 (-1.2, 6.0)	0.22
Eotaxin (pg/ml)	13	51 (44, 69)	63 (52, 108)	17 (-10, 56)	0.11
IL-5 (pg/ml)	13	2.6 (0.5, 4.8)	2.5 (0.9, 8.6)	0.3 (-1.4, 4.2)	0.33
Urine tests					
FDP(+/-) (**)	13	4 (31%)	2 (15%)	-15% (-52%, 21%)	0.63
MMP activity (FU)	3	0.0015 (0.0009, 0.0018)	0.0005 (0.0002, 0.0014)	-0.0004 (-0.0016, 0.0000)	0.10
GAG:creatinine (µg/mmol)	2	*insufficient*	*data*		

(*) Mean (standard deviation) reported, along with mean change (95% CI)

(**) Number (%) with positive responses reported, along with percentage change (95% CI)

The results suggested no changes from exacerbation to recovery in those study subjects with a delayed recovery. However, it is noted that the numbers of patients in the analyses are relatively small.

3.3.3 Eosinophils, platelets, PF4 and Eotaxin

There is clinical significance in the relationship between eosinophils and eotaxin, platelets and PF4, and eosinophils and platelets. The following analyses examined the strength of the association between these variables. All variables were measured on a continuous scale, and several of the variables had skewed distributions. As a result, Spearman's rank correlation was used for all analyses.

Results

The correlation between platelets and eosinophils, platelets and PF4 and eotaxin and eosinophils were examined at each study visit. Examination of an association between variables was only possible where data was present for both comparators at the same study visit. The data is summarised in table 10 below, where N denotes the number of participants who had paired data at each study visit alongside their correlation coefficient and significance is denoted by p-value.

Table 10 – Part 1 admission and follow up visits, correlation for platelets, eosinophils, eotaxin and PF4

Timepoint	Variable 1	Variable 2	N	Correlation Coefficient	P-value
Admission	Platelets	Eosinophils	82	0.28	**0.01**
	Platelets	PF4	57	0.30	**0.02**
	Eosinophils	Eotaxin	60	0.51	**<0.001**
Follow-up 1	Platelets	Eosinophils	32	-0.07	0.69
	Platelets	PF4	27	0.28	0.15
	Eosinophils	Eotaxin	27	-0.01	0.95
Follow-up 2	Platelets	Eosinophils	9	0.48	0.19
	Platelets	PF4	7	-0.23	0.61
	Eosinophils	Eotaxin	7	0.76	0.05

There were significant positive correlations at admission between eosinophils and eotaxin, platelets and eosinophils, and platelets and PF4. Although statistically significant the observed correlations were relatively weak with the exception of eosinophils and eotaxin.

No significant correlations were observed at either follow up timepoint, however, less paired data were available for comparison at these follow up visits due to reduced blood sampling from study participants as discussed in Chapter 2.

3.3.4 Urine dilution and FDP result

The dilution of urine samples collected during the study may have an effect on urine analyte results. Urinary creatinine was measured in order to normalise urine concentration during subsequent analyses. MMP activity was measured rather than absolute values. GAG analyses above utilised GAG:creatinine ratio for this purpose. FDP results were binary so further analyses were performed comparing the urine creatinine in patients with and without an FDP at each timepoint. The urine creatinine values were found to have a positively skewed distribution, and thus the Mann-Whitney test was used for the analyses.

Results – urine dilution and FDP Part 1

A comparison of the urine creatinine values was made between patients who were FDP negative and positive, and the results are summarised in table 11 below. The figures presented are the number of patients in each group, along with the median and inter-quartile range urine creatinine values. P values are presented to denote significance.

Table 11 – Part 1 urine FDP results and urine dilution					
Timepoint	FDP negative		FDP positive		P-value
	N	median (IQR)	N	median (IQR)	
Admission	57	9.0 (4.8, 13.0)	13	7.0 (5.0, 19.0)	0.90
Follow-up 1	43	12.8 (4.0, 6.5)	8	6.0 (4.0, 6.5)	0.10
Follow-up 2	18	10.4 (5.0, 14.4)	2	10.7 (2.0, 19.3)	0.90
Recovery	37	10.5 (5.1, 17.0)	9	6.0 (4.0, 7.0)	0.24

The results suggested no evidence of a difference in the urine creatinine values in patients with FDP negative and positive results.

There was a trend towards urine creatinine values being lower and hence urine being more dilute in FDP positive patients at first follow-up, but the result was not statistically significant.

These results suggest the dilution of urine does not have a significant impact on whether FDP is detectable when present in urine. This is an important observation for the development of a future clinical test, particularly if used in point-of-care testing.

3.4 Inflammatory phenotype comparison

Alongside their categorisation by asthma severity, study participants can also be categorised by inflammatory phenotype into eosinophilic or non-eosinophilic subgroups.

Eosinophilia and rate of recovery

A comparison was made to examine whether a relationship exists between eosinophilic status and rate of recovery from asthma exacerbation. Rate of recovery was defined in terms of rapid and delayed as described as above. Eosinophilic phenotype was determined as described by Schleich and colleagues (160) and is discussed in Chapter 5. Due to the categorical nature of both variables,

the analysis was performed using Fisher's exact test, and the results are presented in table 12 below.

Table 12 – Part 1 eosinophilia and rate of recovery			
Recovery	Non-Eosinophilic N (%)	Eosinophilic N (%)	P-value
Rapid Delayed Never	16 (59%) 9 (33%) 2 (7%)	25 (68%) 5 (14%) 7 (19%)	0.12

No significant difference in rate of recovery was observed between the two groups.

Blood and urine markers in eosinophilic and non-eosinophilic subgroups

Further sub-group analysis was performed repeating examinations of the analysed study parameters but comparing eosinophilic with non-eosinophilic participants. Only those patients who recovered were included. The majority of variables were measured on a continuous scale. The statistical methods used were dependent on the distribution of the variables. Where the values were normally distributed, the paired t-test was used to compare exacerbation and recovery. A large number of the variables were not normally distributed, and the Wilcoxon matched-pairs test was preferred. One variable was measured on a binary scale and the paired exact test was used in this case to compare exacerbation and recovery.

Changes from exacerbation to recovery – non-eosinophilic patients

Table 13 summarises the results when comparing exacerbation and recovery in non-eosinophilic patients.

Table 13 – Part 1 exacerbation vs recovery in non-eosinophilic patients					
Variable	N	Exacerbation median (IQR) or mean (SD)(*) or number (%)(**)	Recovery median (IQR) or mean (SD)(*) or number (%)(**)	Change exacerbation - recovery median (95% CI)	P-value
Plasma Fibrin markers					
TAFI (ng/ml) (*)	23	6569 (2301)	7468 (2501)	899 (-706, 2503)	0.26
PAI-1 (pg/ml)	23	19500 (13800, 43700)	19800 (10800, 43600)	3700 (-3896, 7790)	0.38
D-dimer (µgFEU/ml)	22	0.18 (0.11, 0.33)	0.22 (0.12, 0.33)	0.01 (-0.07, 0.05)	0.81
Fibrinogen (g/l) (*)	22	2.75 (0.69)	3.05 (0.69)	0.29 (-0.07, 0.65)	0.10
Plats (10*9/l) (*)	6	299 (59)	341 (63)	42 (-12, 95)	0.10
PF4 (µg/ml) (*)	21	1.8 (1.4)	2.5 (2.0)	0.6 (-0.5, 1.7)	0.24
TGF (pg/ml)	23	1378 (753, 2538)	1599 (965, 2198)	-3 (-546, 823)	0.69
VEGF (pg/ml)	23	7.4 (0.0, 32.8)	0.6 (0.0, 38.0)	-8.4 (-48.9, 0.0)	0.13
Eosinophils (10*9/l)	6	0.00 (0.00, 0.10)	0.20 (0.10, 0.23)	0.20 (0.01, 0.20)	**0.03**
Plasma Cytokines					
IL-6 (pg/ml)	23	1.3 (0.0, 3.4)	2.1 (0.0, 4.5)	0.0 (-0.1, 1.2)	0.51
IFN (pg/ml)	23	25 (4, 50)	25 (11, 33)	0 (-14, 4)	0.63
IL-13 (pg/ml)	23	0.0 (0.0, 10.6)	0.0 (0.0, 2.1)	0.0 (-0.8, 0.0)	0.39
RANTES (pg/ml) (*)	23	2837 (1266)	2415 (1050)	-422 (-1047, 203)	0.18
IL-12 P40 (pg/ml)	23	14 (0, 39)	5 (0, 52)	0 (-9, 5)	0.93
IL-17A (pg/ml)	23	0.2 (0.0, 8.8)	2.4 (0.0, 11.3)	0.0 (-2.3, 2.0)	0.82
IL-8 (pg/ml)	23	2.1 (0.9, 4.5)	2.8 (1.5, 4.1)	0.6 (-0.5, 1.2)	0.52
TNF-α (pg/ml)	23	6.2 (4.1, 9.1)	9.3 (5.6, 11.6)	1.7 (0.1, 4.3)	**0.04**
Eotaxin (pg/ml)	23	47 (41, 68)	63 (48, 86)	17 (1, 27)	**0.01**
IL-5 (pg/ml)	23	2.6 (0.9, 4.3)	2.6 (0.8, 6.1)	0.0 (-0.6, 0.5)	0.94
Urine tests					
FDP (+/-) (**)	21	5 (24%)	3 (14%)	-10% (-37%, 18%)	0.69
MMP activity (FU)	3	0.0018 (0.0009, 0.0035)	0.0005 (0.0002, 0.0027)	-0.0008 (-0.0016, -0.0004)	0.11
GAG:creatinine (µg/mmol)	7	0.29 (0.21, 0.54)	0.17 (0.12, 0.71)	-0.09 (-0.46, 0.50)	0.87

(*) Mean (standard deviation) reported, along with mean change (95% CI)

(**) Number (%) with positive responses reported, along with percentage change (95% CI)

The results suggested strong evidence of a change from exacerbation to recovery in only eosinophils, TNF-α and eotaxin, all increasing in value from exacerbation to recovery.

Changes from exacerbation to recovery - Eosinophilic patients

A similar set of analyses was examined to compare exacerbation and recovery for eosinophilic patients, with the results summarised in table 14 below.

Table 14 – Part 1 exacerbation vs recovery in eosinophilic patients					
Variable	N	Exacerbation median (IQR) or mean (SD)(*) or number (%)(**)	Recovery median (IQR) or mean (SD)(*) or number (%)(**)	Change exacerbation - recovery median (95% CI)	P-value
Plasma Fibrin markers					
TAFI (ng/ml) (*) (†)	27	6040 (2683)	6981 (2346)	941 (-302, 2184)	0.13
PAI-1 (pg/ml)	27	13000 (6694, 21700)	12000 (5610, 23000)	-1506 (-2824, 3260)	0.79
D-dimer (µgFEU/ml)	26	0.22 (0.16, 0.38)	0.20 (0.15, 0.28)	0.00 (-0.11, 0.04)	0.44
Fibrinogen (g/l) (*)	26	3.00 (0.64)	2.72 (0.51)	-0.28 (-0.59, 0.02)	0.06
Plats (10*9/l) (*)	20	275 (100)	245 (6+1)	-29 (-63, 5)	0.09
PF4 (µg/ml) (*)	26	2.1 (1.9)	2.0 (1.6)	0.0 (-0.8, 0.7)	0.91
TGF (pg/ml)	27	1767 (811, 3244)	1798 (898, 25244)	-112 (-696, 317)	0.67
VEGF (pg/ml)	27	1.4 (0.0, 28.2)	10.9 (0.0, 37.0)	0.0 (0.0, 11.1)	0.08
Eosinophils (10*9/l)	14	0.05 (0.00, 0.53)	0.35 (0.20, 0.53)	0.10 (-0.03, 0.30)	0.12
Plasma Cytokines					
IL-6 (pg/ml)	25	1.4 (0.0, 4.1)	0.3 (0.0, 2.8)	0.0 (-1.6, 0.0)	0.17
IFN (pg/ml)	25	24 (6, 54)	19 (9, 24)	-3 (-16, 2)	0.10
IL-13 (pg/ml)	25	0.0 (0.0, 2.9)	0.0 (0.0, 1.6)	0.0 (-0.2, 0.0)	0.08
RANTES (pg/ml) (*)	25	2791 (1287)	2405 (1119)	-386 (-931, 159)	0.16
IL-12 P40 (pg/ml)	25	8 (0, 45)	7 (0, 40)	0 (-19, 5)	0.59
IL-17A (pg/ml)	25	4.4 (0.2, 14.4)	2.7 (0.3, 8.2)	0.0 (-3.7, 1.0)	0.37
IL-8 (pg/ml)	25	2.5 (1.3, 6.9)	2.5 (1.8, 5.1)	-0.4 (-2.5, 1.0)	0.25
TNF-α	25	6.4 (4.3, 12.6)	7.0 (4.6, 13.1)	-0.8 (-1.8, 1.0)	0.49
Eotaxin (pg/ml)	25	53 (38, 64)	57 (49, 86)	6 (2, 24)	**0.02**
IL-5 (pg/ml)	25	3.0 (1.2, 8.4)	1.9 (1.3, 4.6)	-0.4 (-3.1, 0.2)	0.12
Urine tests					
FDP (+/-) (**)	22	4 (18%)	6 (27%)	9% (-20%, 36%)	0.73
MMP activity (FU)	4	0.0015 (0.0010, 0.0016)	0.0011 (0.0003, 0.0018)	-0.0003 (-0.0008, 0.0005)	0.27
GAG:creatinine(µg/mmol)	10	0.47 (0.31, 0.66)	0.35 (0.22, 0.51)	-0.18 (-0.47, 0.26)	0.44
Additional tests					
FeNO (ppb)	30	57 (24, 94)	40 (28, 74)	-3 (-34, 17)	0.33

(*) Mean (standard deviation) reported, along with mean change (95% CI)

(**) Number (%) with positive responses reported, along with percentage change (95% CI)

(†) Omitting one patient with extremely high value at follow-up

Only eotaxin changed significantly from exacerbation to recovery in eosinophilic patients, demonstrating an increase at recovery.

3.5 Clinical parameters – Part 1

In order to provide objective measures of clinical recovery and to investigate whether clinical measurements used in practice were statistically justified markers of asthma exacerbation, FeNO, FEV_1 and PEFR was compared between exacerbation and recovery. Those participants who did not recover during the study period were excluded.

Where the values were normally distributed, the paired t-test was used to compare between timepoints, and the mean and standard deviation at each visit is reported alongside the mean difference and corresponding confidence interval. Where variables are not normally distributed, the Wilcoxon matched-pairs test was used and the median and inter-quartile range at each timepoint is reported, along with the median difference and corresponding confidence interval. P-values indicating the significance of the results are also given. The results are summarised in table 15 below.

Table 15 – Part 1 clinical markers at exacerbation and clinical recovery

Variable	N	Exacerbation median (IQR) or mean (SD)(*)	Recovery median (IQR) or mean (SD)(*)	Change exacerbation - recovery median (95% CI)	P-value
FeNO (ppb)	54	25 (13, 62)	24 (16, 47)	-1 (-5, 6)	0.65
FEV1 (l) (*)	50	1.96 (0.77)	2.54 (0.72)	0.58 (0.43, 0.72)	**<0.001**
PEFR (l/min) (*)	55	269 (112)	389 (111)	120 (87, 153)	**<0.001**

(*) Mean (standard deviation) reported, along with mean change (95% CI)

There was no significant change in FeNO, but FEV1 and PEFR significantly increased from exacerbation to recovery. There was a mean increase of 0.6 litres in FEV1 and 120 l/min in PEFR between the two timepoints.

FeNO in eosinophilic and non-eosinophilic subgroups

FeNO is a surrogate marker of airway eosinophilic inflammation and therefore specific subgroup examination was made of this marker comparing eosinophilic (table 16) and non-eosinophilic (table 17) subjects at exacerbation and at clinical recovery.

Table 16 – Part 1 FeNO exacerbation vs recovery in eosinophilic asthma

Variable	N	Exacerbation median (IQR)	Recovery median (IQR)	Change exacerbation - recovery median (95% CI)	P-value
FeNO (ppb)	30	57 (24, 94)	40 (28, 74)	-3 (-34, 17)	0.33

Table 17 – Part 1 FeNO exacerbation vs recovery in non-eosinophilic asthma

Variable	N	Exacerbation median (IQR)	Recovery median (IQR)	Change exacerbation - recovery median (95% CI)	P-value
FeNO (ppb)	24	14 (10, 22)	17 (13, 22)	3 (-4, 8)	0.37

When comparing FeNO at exacerbation with recovery, there was no significant difference observed in eosinophilic or non-eosinophilic inflammatory phenotypes.

4.0 PART 2 RESULTS

4.1 Part 2 screening, recruitment, withdrawal and completion

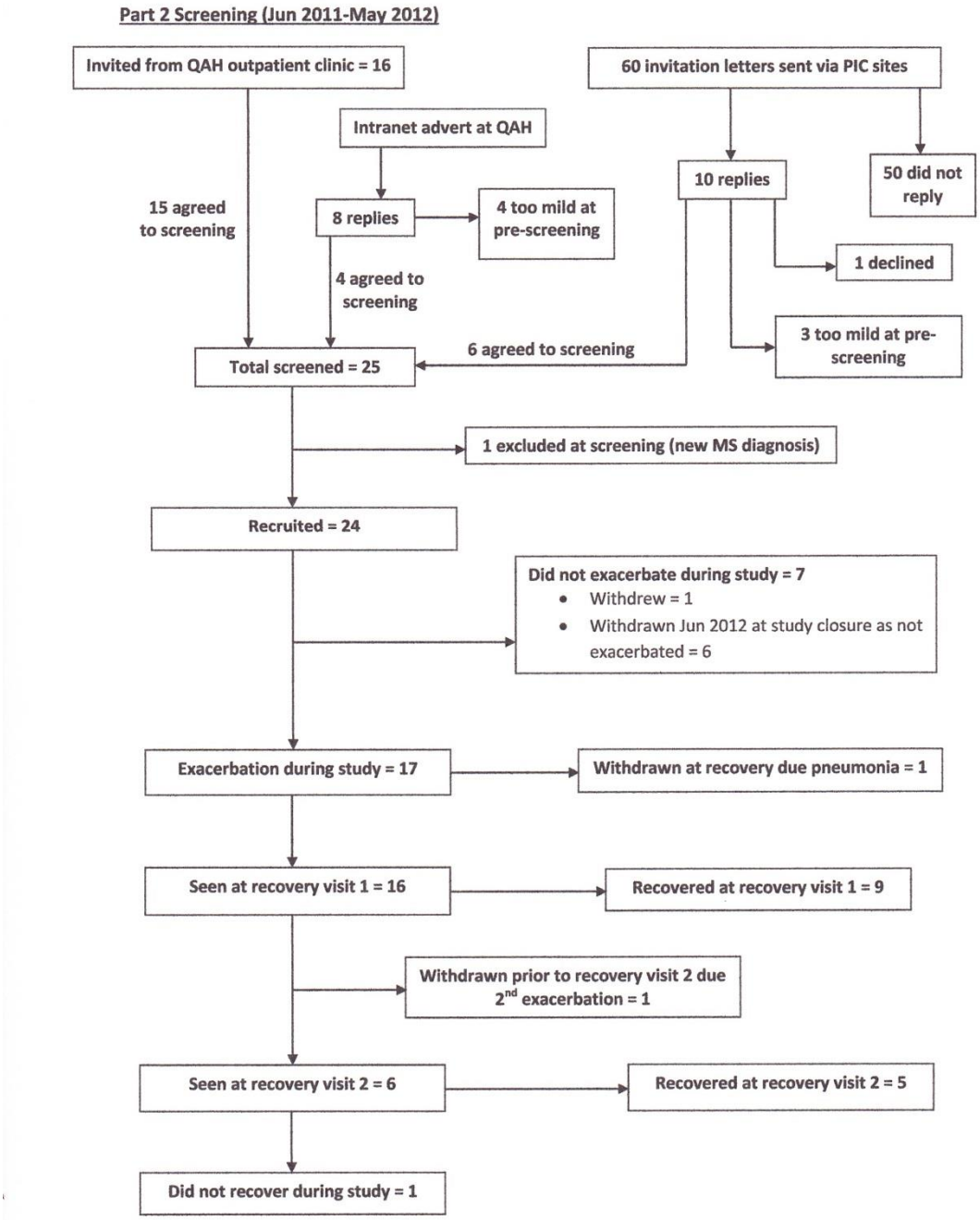
Part 2 successfully recruited 24 patients with stable asthma, 15 of whom were followed up until acute exacerbation and through to subsequent recovery. This is the largest prospective study to date examining coagulation and fibrinolysis in asthma both in stable and exacerbation states.

Screening and recruitment to Part 2 is summarised in figure 8 below. 25 patients were eventually screened and 24 recruited, of which 6 had moderate and 18 severe asthma by study criteria. Only 1 participant withdrew after several months of study involvement and prior to an asthma exacerbation, this participant had severe asthma. Of the remaining 23, 6 had not exacerbated by the time of study closure in June 2012 and were withdrawn, 4 were moderate and 2 severe.

Of the 17 remaining participants, 1 severe asthmatic experienced pneumonia at the time of exacerbation and was hence withdrawn and 1 had a second exacerbation at the time of the second recovery visit and was then withdrawn.

15 participants completed the study, 9 of who recovered at recovery visit 1, 5 recovered at recovery visit 2 and 1 did not recover during the study period. See Figure 8 below for a summary of Part 2 study recruitment and screening.

Figure 8 – Part 2 screening



4.1.1 Part 2 adverse events

There was 1 adverse event from the Part 2 population during the study period, which was a SAE due to the participant being admitted to hospital.

Table 18 – Part 2 adverse events

Study number	Adverse event	Related to study activity?
J2004XX*	Re-admission to hospital with asthma	No

*initials removed to preserve anonymity

4.2 Part 2 participant characteristics

Patient characteristics were summarised for all patients in Part 2 of the study. Continuous variables were summarised by both the mean and standard deviation, if found to be normally distributed, or median and inter-quartile range if not normally distributed. Categorical variables were summarised by the number and percentage of patients in each category.

Comparisons were made between patients who did and did not complete the study. The analysis of continuous variables found to be normally distributed was performed using the unpaired t-test, whilst continuous variables not found to be normally distributed were analysed using the Mann-Whitney test. Binary categorical variables and those with no ordering of the categories were analysed using Fisher's exact test. Ordinal categorical variables were analysed using the Mann-Whitney test (in order to take account of the order of the categories, which is not utilised using Fisher's exact test).

Results

A summary of the patient characteristics is given in the two subsequent tables (19 and 20). The figures reported are the results for all patients, and then split by those who withdrew from the study and those who did not. P-values indicating the significance of the difference between those who did and did not withdraw are also presented.

Table 19 – Participant characteristics Part 2 continuous variables				
Variable	All patients (n=24)	Not withdrawn (n=15)	Withdrawn (n=9)	P-value
Age (years) (*)	47.1 (12.6)	48.8 (13.9)	44.3 (10.1)	0.41
Hospital admissions (**)	0 (0, 1)	0 (0, 2)	1 (0, 1)	0.86
BMI (kg/m^2) (**)	28 (25, 33)	29 (24, 32)	27 (26, 33)	0.68

(*) Mean (standard deviation) reported

(**) Median (inter-quartile range) reported

The results suggested no significant differences been those who did and didn't withdraw for the three continuous variables examined.

Table 20 – Participant characteristics Part 2 categorical variables					
Variable	Category	All patients (n=24)	Not withdrawn (n=15)	Withdrawn (n=9)	P-value
Sex	Female	20 (83%)	12 (80%)	8 (89%)	1.00
	Male	4 (17%)	3 20%)	1 (11%)	
GINA 2008	Intermittent	4 (17%)	2 (13%)	2 (22%)	**0.04**
	Mild Persist.	6 (25%)	2 (13%)	4 (44%)	
	Mod. Persist	4 (17%)	2 (13%)	2 (22%)	
	Severe Persist	10 (42%)	9 (60%)	1 (11%)	
Asthma Severity	Moderate	6 (25%)	2 (13%)	4 (44%)	0.15
	Severe	18 (75%)	13 (87%)	5 (56%)	
Eosinophilic	No	9 (37%)	3 (20%)	6 (67%)	**0.04**
	Yes	15 (63%)	12 (80%)	3 (33%)	
Smoking status	Current	0 (0%)	0 (0%)	0 (0%)	0.15
	Ex	10 (83%)	7 (100%)	3 (60%)	
	Never	2 (17%)	0 (0%)	2 (40%)	
Obese	No	13 (54%)	8 (53%)	5 (56%)	1.00
	Yes	11 (46%)	7 (47%)	4 (44%)	
Atopy (SPT positive)	No	5 (24%)	5 (33%)	0 (0%)	0.26
	Yes	16 (76%)	10 (67%)	6 (100%)	
Atopy (raised IgE)	No	8 (38%)	5 (33%)	3 (50%)	0.63
	Yes	13 (62%)	10 (67%)	3 (50%)	
Recovery	Rapid	-	9 (60%)	-	-
	Delayed	-	5 (33%)	-	
	Never	-	1 (7%)	-	
	DNA	-	-	4 (44%)	
	Withdrawn	-	-	5 (56%)	

There were significant differences between participants who completed the study and those who withdrew when looking at asthma severity and eosinophilic inflammatory status. Those participants who withdrew had less severe asthma and those who completed the study had more eosinophilic inflammation.

Table 21 – Part 2 time from recruitment to exacerbation

Study number	Days
2001XX	67
2002XX	13
2003XX	83
2006XX	74
2009XX	270
2010XX	31
2011XX	8
2012XX	54
2013XX	28
2014XX	15
2015XX	53
2016XX	98
2017XX	52
2018XX	28
2023XX	42

XX Initials removed to preserve anonymity

The number of days from study recruitment to asthma exacerbation in Part 2 is listed in table 21 above. The time from recruitment to exacerbation is 61 days or 46 days if the outlier 2009XX is excluded.

Discussion

Recruitment to Part 2 of the study was only from the QAH catchment population. By definition, the population was narrowed by the inclusion criteria requiring subjects to have either moderate or severe asthma defined by inhaled steroid doses (158). As with Part 1 the majority of the study population was female (83%), eosinophilic (63%) and atopic (76% by skin prick test, 62% by elevated IgE). Again it

was not formally recorded on the CRF but ethnicity of the study recruits was predominantly white Caucasian due to the local population. As discussed in chapter 2, recruitment of subjects with moderate asthma was more difficult than those with severe resulting in 75% of Part 2 subjects having severe asthma. This will be discussed in more detail below.

In contrast to Part 1 of the study, those who were withdrawn or withdrew from Part 2 were in the main (78%) those who did not exacerbate and hence more stable. This is consistent with our finding that the group who withdrew had less severe asthma and hence were less likely to exacerbate. Furthermore, those who did complete the study (and hence exacerbated) were more likely to be eosinophilic and therefore more prone to exacerbate (32).

It is worth noting that overall, the population who completed Part 2 was very small (15 subjects). Clearly this will have an impact on the statistical strength of the results from this population. Again, the factors discussed here should be taken into consideration when extrapolating findings onto other populations.

4.3 Part 2 plasma analyses

Patients in part 2 of the study were seen on a first visit, at exacerbation, and then potentially at two subsequent follow-up timepoints. Attention was focussed on a comparison between two pre-determined sets of values at visits with clinical relevance.

The first set of analyses compared the differences in values between the first visit (baseline) and values at exacerbation.

A second set of analyses examined the changes from the baseline first visit to the patient recovery. Data from the first follow-up timepoint (30 days from exacerbation) was used for those who rapidly recovered, whilst data from the second follow-up timepoint (61 days from exacerbation) was used for those with delayed recovery. Patients who did not recover were omitted from the analyses.

All outcome variables were measured on a continuous scale. The statistical methods used were dependent on the distribution of the variables. Where the values were normally distributed, the paired t-test was used to compare between timepoints. For a large number of the variables the values were not normally distributed, and the Wilcoxon matched-pairs test was preferred.

4.3.1 Part 2 clinical comparison results

The first set of analyses examined the change in values from the first visit to exacerbation, and a summary of the results is given in the subsequent table (table 22). The first figures reported are the number of patients with measurements at both timepoints. For those variables where the values were not normally distributed, the median and inter-quartile range at each timepoint is reported, along with the median difference and corresponding confidence interval. For those variables where the values were normally distributed, the mean and standard deviation at each timepoint is reported, along with the mean difference and corresponding confidence interval. P-values indicating the significance of the results are also given.

Table 22 – Part 2 plasma baseline vs exacerbation					
Variable	N	Baseline median (IQR) or mean (SD)(*)	Exacerbation median (IQR) or mean (SD)(*)	Change baseline - exacerbation median (95% CI)	P-value
Plasma Fibrin markers					
TAFI (ng/ml)(*)	17	5406 (2591)	4764 (2030)	-641 (-1967, 683)	0.32
PAI-1 (pg/ml)	17	15000 (10336, 30100)	16200 (11400, 37850)	2000 (-3634, 9390)	0.33
D-dimer (µgFEU/ml)	14	0.21 (0.12, 0.29)	0.25 (0.20, 0.42)	0.07 (-0.01, 0.15)	**0.02**
Fibrinogen (g/l)(*)	13	3.08 (0.57)	3.21 (0.50)	0.12 (-0.19, 0.43)	0.40
Plats (10^*9/l)(*)	13	305 (61)	318 (64)	13 (-12, 38)	0.28
PF4 (µg/ml) (*)	17	3.9 (2.9)	2.6 (1.6)	-1.4 (-3.1, 0.4)	0.12
TGFβ1 (pg/ml)	17	1801 (721, 2570)	966 (682, 2376)	-666 (-1289, -2)	**0.02**
VEGF (pg/ml)	17	23 (3, 94)	20 (0, 37)	-1 (-17, 3)	0.22
Eosinophils (10^*9/l)	13	0.30 (0.15, 0.50)	0.30 (0.20, 0.85)	0.00 (-0.10, 0.38)	0.28
Plasma Cytokines					
IL-6 (pg/ml)	15	0.0 (0.0, 1.4)	1.2 (0.0, 3.2)	0.6 (0.0, 2.3)	0.19
IFN (pg/ml)	15	22 (8, 43)	16 (8, 51)	3 (-18, 22)	0.61
IL-13 (pg/ml)	15	0.0 (0.0, 14.6)	0.0 (0.0, 3.8)	0.0 (-4.3, 0.0)	0.43
RANTES (pg/ml) (*)	15	2803 (1148)	2433 (944)	-371 (-1059, -318)	0.27
IL-12 P40 (pg/ml)	15	14 (0, 49)	7 (0, 38)	0 (-13, 5)	0.45
IL-17A (pg/ml)	15	1.9 (0.0, 4.5)	0.4 (0.0, 8.5)	0.0 (-1.8, 5.2)	0.75
IL-8 (pg/ml)	15	3.5 (1.0, 6.8)	2.6 (0.6, 4.8)	0.0 (-4.0, 1.5)	0.53
TNF-α (pg/ml)	15	9.1 (3.9, 10.3)	5.7 (3.5, 12.9)	-1.1 (-4.2, 2.7)	0.69
Eotaxin (pg/ml)	15	65 (47, 96)	61 (41, 74)	-15 (-38, 6)	0.10
IL-5 (pg/ml)	15	1.6 (0.0, 4.9)	2.2 (0.0, 6.0)	0.6 (-1.8, 3.8)	0.39

(*) Mean (standard deviation) reported, along with mean change (95% CI)

Only D-dimer and TGFβ1 significantly changed from baseline to exacerbation.

There was a significant increase in D-dimer at exacerbation with a median increase

of 0.07 µgFEU/ml. Conversely there was a significant decrease in TGFβ1 at exacerbation, with a median decrease of over 650 pg/ml.

Additionally, there was very weak evidence of a decrease in eotaxin from baseline to exacerbation, but this result was not quite statistically significant.

A similar set of analyses were performed to examine the change in the various parameters from baseline to recovery and a summary of the results is given in the subsequent table (table 23).

Table 23 – Part 2 plasma baseline vs recovery					
Variable	N	Baseline median (IQR) or mean (SD)(*)	Recovery median (IQR) or mean (SD)(*)	Change baseline - recovery median (95% CI)	P-value
Plasma Fibrin markers					
TAFI (ng/ml)(*)	14	5596 (2799)	5994 (1921)	398 (-1467, 2263)	0.65
PAI-1 (pg/ml)	14	16200 (12000, 33425)	16350 (13750, 22325)	1211 (-6562, 3291)	0.64
D-dimer (µgFEU/ml)	14	0.18 (0.09, 0.27)	0.21 (0.11, 0.36)	0.03 (0.00, 0.08)	0.08
Fibrinogen (g/l)(*)	12	2.91 (0.48)	3.04 (0.54)	0.13 (-0.29, 0.56)	0.50
Plats (10^*9/l)(*)	14	293 (63)	318 (110)	24 (-13, 61)	0.18
PF4 (µg/ml) (*)	14	3.9 (3.0)	2.2 (0.8)	-1.7 (-3.4, -0.1)	**0.04**
TGFβ1 (pg/ml)	14	1425 (779, 2111)	1193 (565, 2310)	-132 (-936, 236)	0.27
VEGF (pg/ml)	14	21 (1, 101)	9 (0, 46)	-3 (-5, 1)	0.13
Eosinophils (10^*9/l)	14	0.30 (0.10, 0.43)	0.20 (0.10, 0.50)	0.00 (-0.20, 0.10)	0.61
Plasma Cytokines					
IL-6 (pg/ml)	13	0.0 (0.0, 1.9)	0.0 (0.0, 1.5)	0.0 (0.0, 0.2)	0.37
IFN (pg/ml)	13	22 (7, 45)	21 (2, 37)	-5 (-14, 4)	0.16
IL-13 (pg/ml)	13	0.0 (0.0, 10.3)	0.0 (0.0, 1.9)	0.0 (-1.5, 0.0)	0.16
RANTES (pg/ml) (*)	13	2861 (1215)	2265 (850)	-596 (-1356, 163)	0.11
IL-12 P40 (pg/ml)	13	14 (0, 45)	0 (0, 32)	-7 (-14, 0)	0.05
IL-17A (pg/ml)	13	2.5 (0.0, 5.4)	2.5 (0.0, 5.3)	0.0 (-1.2, 0.0)	0.28
IL-8 (pg/ml)	13	3.4 (0.5, 6.0)	2.2 (0.0, 4.5)	0.0 (-2.3, 0.5)	0.31
TNF-α (pg/ml)	13	6.4 (3.9, 10.1)	5.0 (3.7, 8.7)	-0.9 (-3.7, 1.6)	0.31
Eotaxin (pg/ml)	13	65 (43, 105)	60 (50, 88)	5 (-43, 14)	0.97
IL-5 (pg/ml)	13	1.4 (0.0, 5.6)	0.9 (0.0, 3.9)	-0.2 (-1.8, 0.6)	0.38

(*) Mean (standard deviation) reported, along with mean change (95% CI)

PF4 significantly decreased from baseline to recovery, with a mean decrease of 1.7 µg/ml between the two timepoints.

There was some evidence of a reduction in IL-12 values over the same time period, with a median decrease of 7 pg/ml. This reduction was only of borderline statistical significance. There was also weak evidence that the D-dimer values were higher at recovery than at visit 1, but again the result did not reach statistical significance.

Changes from baseline to recovery were not found to be significant in the other parameters examined.

4.3.2 Part 2 plasma baseline through exacerbation to recovery

Patients in part 2 of the study were measured on a first visit (baseline), at exacerbation, and then potentially at two subsequent follow-up timepoints. Data from the last timepoints was combined to give recovery data. The first follow-up timepoint (30 days) was used for those who recovered rapidly, whilst data from the second follow-up timepoint (61 days) was used for those with delayed recovery, giving a linear comparison from baseline through exacerbation to subsequent clinical recovery. Patients who did not recover were omitted from these analyses.

Data from all three timepoints were included in the analysis. For variables that were normally distributed, the analysis was performed using two way ANOVA, with the two factors being patient and time. The patient differences were not of interest, but were included to allow for repeat measurements from the same patient. For a large number of the variables the values were not normally distributed, and the Friedman test was preferred for these measures.

Results

The analyses examined the change in values over the three timepoints, and a summary of the results is given in table 24. For the non-normally distributed, the median and inter-quartile range at each timepoint are reported, whilst for normally distributed variables the mean and standard deviation at each timepoint are reported. P-values indicating the significance of the overall difference between timepoints are also reported.

Table 24 – Part 2 plasma linear progression from baseline through exacerbation to recovery

Variable	N	Baseline median (IQR) or mean (SD)(*)	Exacerbation median (IQR) or mean (SD)(*)	Recovery median (IQR) or mean (SD)(*)	P-value
Plasma Fibrin markers					
TAFI (ng/ml)(*)	14	5596 (2799)	4966 (2095)	5994 (1921)	0.42
PAI-1 (pg/ml)	14	16200 (12300, 31800)	19500 (11000, 38200)	16350 (14400, 21000)	0.69
D-dimer (µgFEU/ml)	11	0.19 (0.10, 0.29)	0.23 (0.18, 0.34)	0.19 (0.13, 0.38)	0.07
Fibrinogen (g/l)(*)	12	2.91 (0.48)	3.22 (0.53)	3.03 (0.52)	0.65
Plats (10^*9/l) (*)	14	293 (63)	311 (61)	318 (110)	0.39
PF4 (µg/ml) (*)	14	3.9 (3.0)	2.6 (1.7)	2.2 (0.8)	0.08
TGFβ1 (pg/ml)	14	1425 (780, 2111)	894 (638, 1939)	1193 (565, 2310)	0.12
VEGF (pg/ml)	14	21 (1, 101)	14 (0, 32)	9 (0, 46)	0.50
Eosinophils (10^*9/l)	12	0.30 (0.13, 0.48)	0.30 (0.15, 0.63)	0.20 (0.10, 0.48)	0.89
Plasma Cytokines					
IL-6 (pg/ml)	12	0.0 (0.0, 1.1)	1.6 (0.0, 3.0)	0.0 (0.0, 0.4)	0.11
IFN (pg/ml)	12	18 (7, 43)	14 (8, 48)	13 (1, 33)	0.28
IL-13 (pg/ml)	12	0.0 (0.0, 12.4)	0.0 (0.0, 4.5)	0.0 (0.0, 2.9)	0.48
RANTES (pg/ml) (*)	12	2810 (1183)	2479 (1019)	2265 (850)	0.16
IL-12 P40 (pg/ml)	12	14 (0, 47)	6 (0, 56)	0 (0, 32)	0.05
IL-17A (pg/ml)	12	1.3 (0.0, 4.4)	0.2 (0.0, 8.4)	1.3 (0.0, 4.1)	0.96
IL-8 (pg/ml)	12	3.5 (0.2, 6.3)	2.3 (0.3, 7.2)	1.9 (0.0, 4.7)	0.74
TNF-α (pg/ml)	12	6.2 (3.8, 10.2)	4.5 (3.3, 10.5)	4.8 (3.3, 8.6)	0.65
Eotaxin (pg/ml)	12	65 (44, 110)	53 (40, 71)	62 (50, 94)	0.50
IL-5 (pg/ml)	12	1.4 (0.0, 3.9)	1.3 (0.0, 3.7)	0.5 (0.0, 2.2)	0.65

(*) Mean (standard deviation) reported

The results suggested no strong evidence of a change in values between the three timepoints. However, there was some evidence of a difference between timepoints for D-dimer, PF4 and IL-12, although these results were only of borderline statistical significance.

D-dimer values increased from baseline to exacerbation, and then decreased again at recovery, to similar levels observed at visit 1. Both PF4 and IL-12 showed a steady decrease over the three timepoints from baseline, through exacerbation to recovery.

4.4 Part 2 urine analyses

In Part 2 of the study, alongside the study visits, participants prospectively collected urine samples allowing linear comparison of urine results in relation to clinical exacerbation.

4.4.1 Part 2 FDP in relation to exacerbation

The association between FDP and exacerbation was examined by splitting the observation time for each patient into time intervals. Various lengths of time interval were used from 4 to 15 days. The intervals were so created so that a positive FDP result occurred at the start of each interval, and so the interval lengths considered ranged from FDP+3 days up to FDP+14 days. The occurrence of an exacerbation in intervals starting with a positive FDP were compared to the occurrence of exacerbation in other time intervals with no positive FDP. Data after exacerbation was omitted from the analysis, and only whole intervals were included in the analysis. Days that only formed part of an interval were omitted.

A feature of this data is that each patient contributed several time intervals. Therefore to allow for the repeat measurements from each patient the analysis was performed using multilevel statistical methods. Two-level models were used with individual time intervals contained within patients. Due to the binary nature of the

outcome (i.e. exacerbation, yes/no), the analysis was performed using multilevel logistic regression.

Results of the analyses using different time window widths are summarised in table 25 below. The first column represents the number days that comprise each time window. The second and third columns outline the occurrence of the pre-determined time window beginning with either a negative or positive urine FDP, and the number and percentage of each occurrence. The size of difference between groups was summarised as an odds ratio, giving the odds of exacerbation with a positive FDP result relative to the odds of no exacerbation with a negative FDP result. P-values indicating the significance of the results are also reported.

Table 25 – Part 2 urine FDP related to exacerbation

Time Window	Positive or negative FDP	Number of time windows (%)	Odds Ratio (95% CI)	P-value
4 days (FDP+3days)	No FDP	15/231 (6%)	1	0.21
	FDP	2/12 (17%)	2.86 (0.56, 14.6)	
8 days (FDP+7days)	No FDP	13/112 (12%)	1	**0.03**
	FDP	4/11 (36%)	4.35 (1.12, 16.9)	
11 days (FDP+10days)	No FDP	12/78 (15%)	1	**0.02**
	FDP	5/10 (50%)	5.50 (1.38, 21.9)	
15 days (FDP+14days)	No FDP	11/56 (20%)	1	**0.02**
	FDP	6/10 (60%)	6.07 (1.43, 25.8)	

There was a significantly increased occurrence of exacerbation after a positive FDP result using window widths of 8, 11 and 15 days.

For example using an interval length of 11 days, 15% of all intervals with no positive FDP contained exacerbation, compared to 50% of all intervals starting with a

positive FDP result. The odds of exacerbation in the 10 days after a positive FDP result exacerbation were 5.5 times greater than at other times.

4.4.2 Part 2 FDP and urine dilution

Analyses comparing the urine creatinine in samples with positive and negative FDP results were performed to examine whether urine dilution affects FDP result. The urine creatinine values were found to have a positively skewed distribution, and thus the Mann-Whitney test was used for the analyses.

A comparison of the urine creatinine values was made between measurements that were FDP negative and positive, and the results are summarised in table 26 below. The figures presented are the number of patients in each group, along with the median and inter-quartile range creatinine values.

Table 26 – urine creatinine and FDP

FDP	Number of patients	Creatinine (nmol/ml) Median (IQR)	P-value
Negative	412	10.1 (6.1, 15.4)	0.39
Positive	22	11.1 (8.1, 15.5)	

The results suggested no evidence of a difference in the urine creatinine values in samples that were FDP negative and positive results. Hence urine dilution had no effect on the likelihood of a positive or negative FDP result.

4.4.3 Urine GAG analysis

Analyses examined if the GAG:creatinine ratio was higher or lower in the period immediately preceding an exacerbation, relative to the previous time period. The first interval considered was the day of exacerbation plus the one day before exacerbation. The mean GAG:creatinine ratio in this period was calculated for each patient and compared to the mean value in the days preceding this period. The

analysis was repeated this time considering exacerbation plus the preceding five days, and also exacerbation plus the preceding seven days. The paired t-test was used to compare the GAG:creatinine ratio in the period before exacerbation to previous values

The paired t-test was used to compare the GAG:creatinine ratio at and immediately before exacerbation to the values on earlier days. A summary of the results is given in table 27 below. The figures reported are the mean and standard deviation values for each interval. P-values indicating the significance of the results are also presented.

Table 27 – GAG:creatinine ratio (µg/mmol) related to exacerbation

Recent Time period	N	Exacerbation + preceding days mean (SD)	Previous days mean (SD)	P-value
Exacerbation + 1 day	12	0.74 (0.58)	0.49 (0.14)	0.15
Exacerbation + 5 days	14	0.62 (0.27)	0.45 (0.20)	0.06
Exacerbation + 7 days	14	0.61 (0.25)	0.45 (0.18)	**0.03**

The results suggested some evidence that GAG:creatinine ratio was higher on the day of exacerbation and earlier days when the 5 and 7 day windows were used, however, the result was only of borderline significance for the 5 day period. Using the 7 day window, there was a significant increase in GAG:creatinine ratio before exacerbation. The mean GAG:creatinine ratio was 0.61 µg/mmol before exacerbation, increased from 0.45 µg/mmol in the preceding period.

4.5 PEFR analyses

PEFR was measured and recorded by Part 2 study subjects prospectively during the study period. Greater diurnal variation on PEFR is considered clinically significant in

terms of asthma instability or exacerbation, as are overall lower values in relation to predicted or best measured.

4.5.1 PEFR related to exacerbation – Part 2

The first set of analyses examined whether PEFR demonstrated significant variability just before exacerbation, compared with the preceding measurements throughout the study. Average daily PEFR values were used for this analysis.

The variation in the peak flow values over a time interval was quantified by calculating the standard deviation of the measurements. The higher the standard deviation the greater the variation in values. The first interval considered was the day of exacerbation plus the one day before exacerbation. The variation in peak flow in this period was calculated for each patient along with the variation in the days preceding this period. The analysis was repeated this time considering peak flow at exacerbation plus the preceding five days, and then days before this time period. The paired t-test was used to compare variation in peak flow before exacerbation to previous variation.

Initially the peak flow values over time for each patient were examined, and a plot of the change in values over time for each patient is given in figure 9.

Figure 9 – PEFR (l/min) over time

The paired t-test was used to compare the variation in peak flow values at and immediately before exacerbation to the variation on earlier days. A summary of the results is given in table 28 below. The figures reported are the mean and standard deviation variation (as measured by the standard deviation over the time period) in each interval. P-values indicating the significance of the results are also presented.

Table 28 - PEFR variability (l/min) immediately before exacerbation relative to preceding days				
Recent Time period	N	Exacerbation + preceding days mean (SD)	Previous days mean (SD)	P-value
Exacerbation + 1 day	12	19.2 (14.7)	22.3 (17.8)	0.65
Exacerbation + 5 days	14	20.5 (13.8)	21.0 (16.5)	0.93

There was no significant difference observed between PEFR in the 5 days immediately prior to exacerbation, compared with the preceding days.

4.5.2 PEFR at exacerbation relative to baseline

Further analyses were performed to examine if the peak flow in the days immediately preceding exacerbation differed compared to peak flow values at baseline. The baseline period was considered to be the first two weeks of measurement. Two different time periods before exacerbation were considered, 7 days or 14 days. The mean peak flow value in each period was calculated, and the paired t-test was used to compare between time periods. One patient had only 15 days of measurements and was excluded from this analysis.

The paired t-test was used for the analysis, and a summary of the results is given in table 29 below. The figures reported are the mean and standard deviation of peak flow in each interval. P-values indicating the significance of the results are also presented.

Table 29 – PEFR (l/min) at baseline relative to PEFR (l/min) before exacerbation				
Pre-exacerbation time window	N	Baseline mean (SD)	Pre-exacerbation mean (SD)	P-value
Exacerbation – 7 days	14	304 (106)	281 (105)	**0.01**
Exacerbation – 14 days	14	304 (106)	284 (99)	**0.01**

When comparing baseline PEFR measured for 2 weeks at the beginning of study enrolment with the pre-exacerbation period, PEFR was significantly lower during the 7 or 14 days pre-exacerbation than at baseline.

While this is useful to demonstrate objective evidence of exacerbation, it is worthy of note that in clinical terms, a mean fall of 20 l/min in PEFR could be due to inter-test variability.

4.6 Symptoms and reliever use – Part 2

Alongside PEFR data clinical symptoms were recorded prospectively by Part 2 study participants. The presence or absence of asthma symptoms during a 24 hour period was recorded alongside the number of times asthma reliever medication was used. Table 30 below outlines the symptoms recorded and the score given for each parameter.

Table 30 – Part 2 symptoms, reliever use and assigned score	
Parameter	**Score**
Wheeze	1
Night waking	1
Chest tightness	1
Breathlessness	1
Reliever use	
≤ 2	0
3 – 9	1
≥ 10	2

When analysing these data, a symptom score from 0-4, the reliever use independent of symptoms and a composite score of symptoms and reliever use together (scored from 0-7) were used.

Data was collected from around 9 months before exacerbation to 2 months post exacerbation in some patients, although the range was less in most patients. One option would be to try and model scores throughout this time period. However, by doing this, the wide range might imply that the important time period around exacerbation was not given sufficient attention. Instead it was decided to focus only on values in the period from 30 days before to 30 days post exacerbation.

All of the three outcome variables of interest (symptom score, composite score, and reliever use) were measured on continuous scales. Reliever use was found to have a positively skewed distribution, and was thus analysed on the log scale.

The change in outcomes over time were modelled. As there were repeat measurements from the same patients during this time period, it was necessary to account for this in the analysis. As a result, all analysis was performed using multilevel linear regression. Two-level models were used with individual scores

nested within patients. To allow a flexible relationship between time and the outcomes, both linear and squared terms for time were included in the analysis (and also a cubic term if this was statistically significant).

Results

Multilevel regression models were used to model the outcomes over time. A summary of the regression coefficients from these models are summarised in table 31 below. These are given as the change in outcome for a one-week increase in time. P-values indicating the overall significance of the change in scores over time are also presented.

Table 31 – Summary of regression coefficients

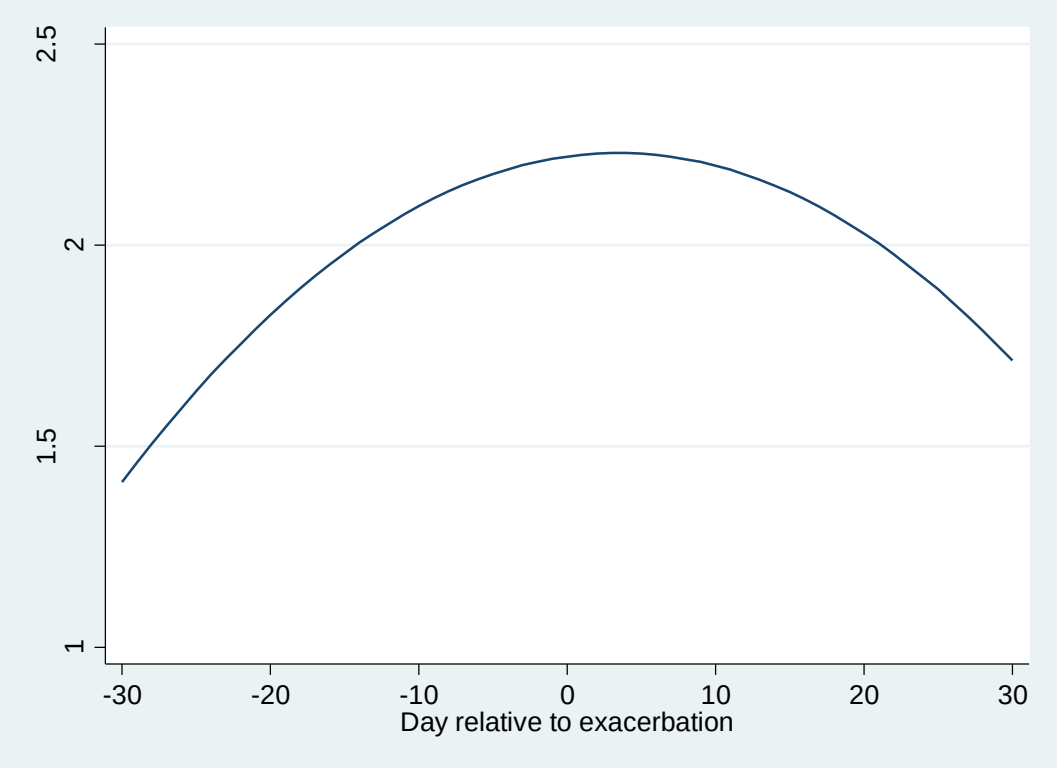
Outcome	Term	Coefficient (95% CI)	P-value
Symptom score	Linear	0.035 (0.001, 0.070)	**<0.001**
	Squared	-0.036 (-0.051, -0.021)	
Composite score	Linear	0.022 (-0.018, 0.063)	**<0.001**
	Squared	-0.045 (-0.064, -0.027)	
Reliever use (*)	Linear	-0.036 (-0.074, 0.001)	**0.002**
	Squared	-0.012 (-0.019, -0.005)	
	Cubic	0.003 (0.000, 0.006)	

(*) Variable analysed on the log scale

The results suggested that there were significant changes in all three outcomes over time. Due the inclusion of squared and cubic terms for time, it is difficult to interpret the nature of the scores over time from these coefficients alone. Therefore, the fitted relationship between time and the scores from the regression models were determined, and the results are shown graphically in the next charts. Additional charts were produced using the mean values of the raw data values at each day.

Figure 10 below, is for symptom score using the fitted regression lines.

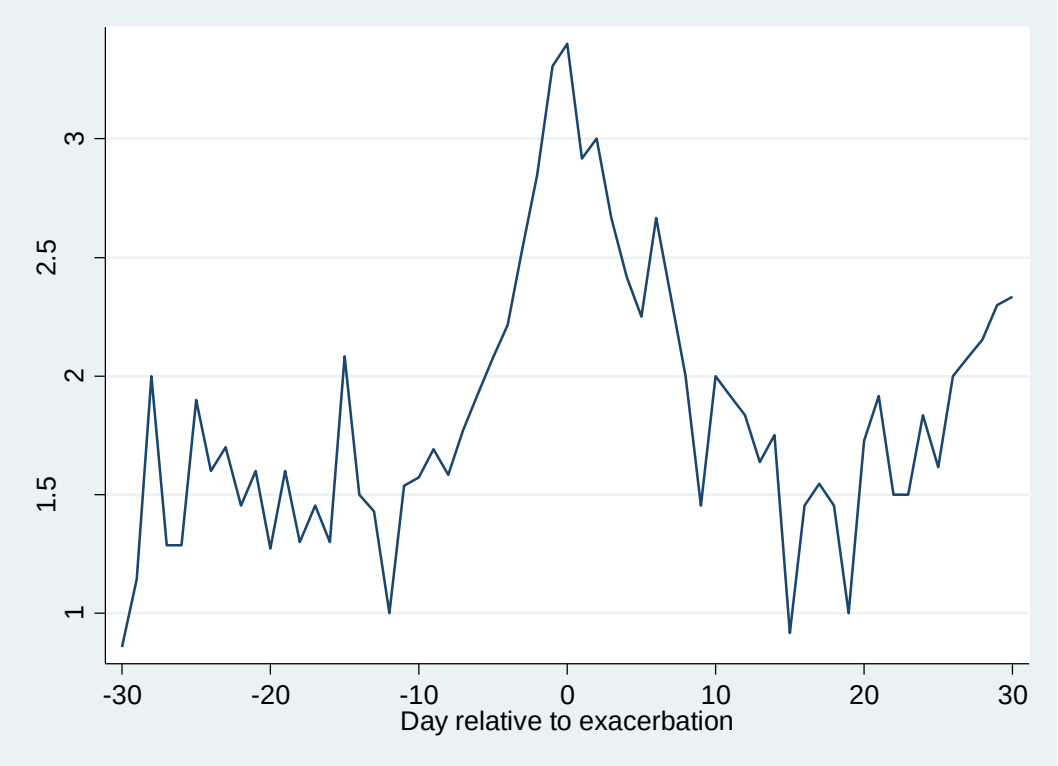
Figure 10 – Symptom score using fitted regression lines



The graph suggests that the peak symptom score was at exacerbation, or just after. Scores increased in the run-up to exacerbation, with a decrease in scores post-exacerbation.

A similar representation of the data using the mean value at each day is shown in the next graph (figure 11).

Figure 11 – Symptom score using mean daily value



The next graph (figure 12) shows the changes over time for the composite score, based on the fitted regression model. This graph shows a similar picture to that for the symptom scores. That is an increase in score preceding exacerbation, with peak values at exacerbation or just after. Again, there was a decrease in scores after exacerbation. Figure 13 demonstrates the changes over time for composite score using raw data.

Figure 12 – Composite score using fitted regression model

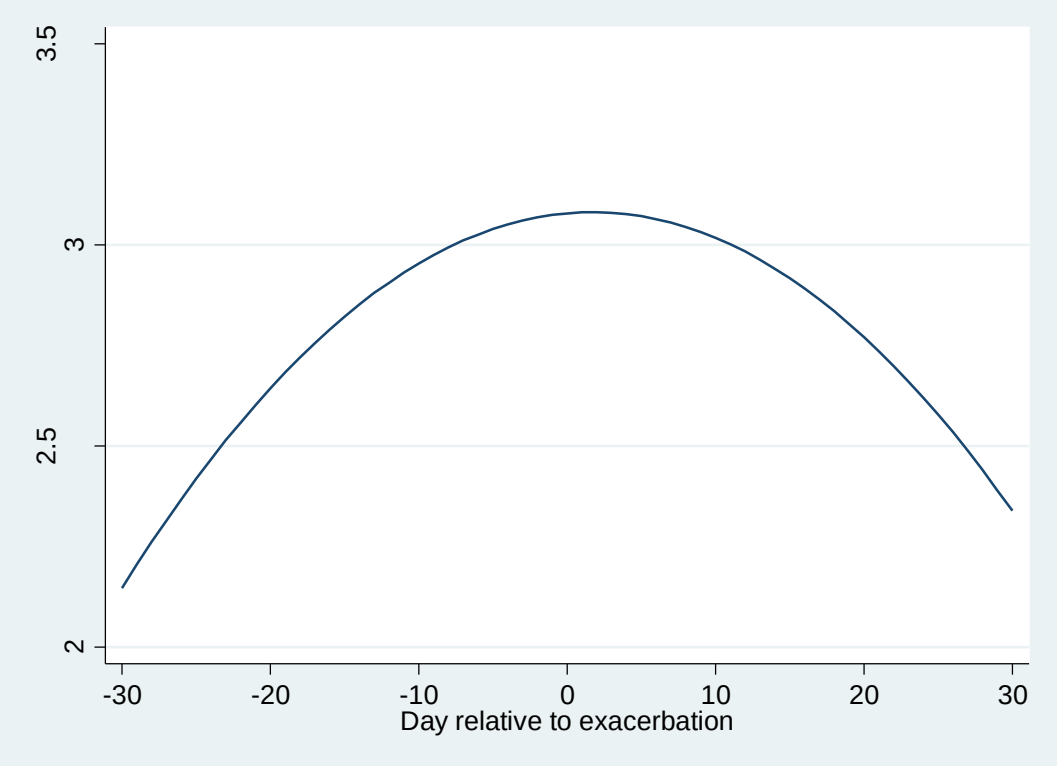
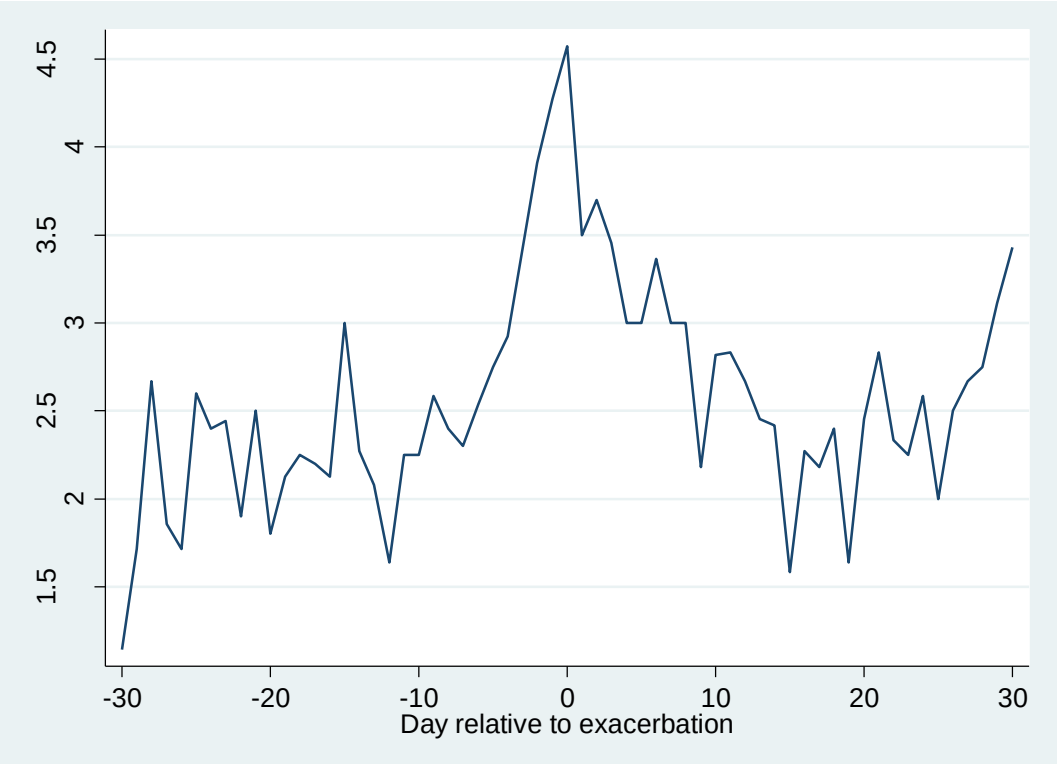
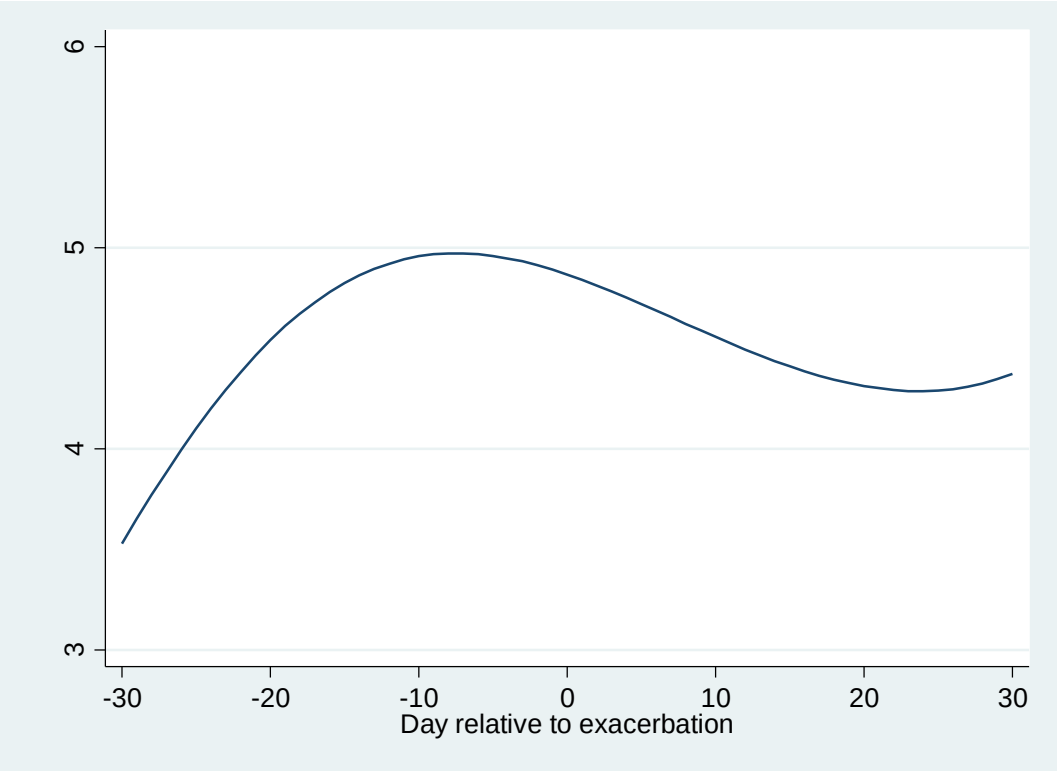


Figure 13 – Composite score using raw data



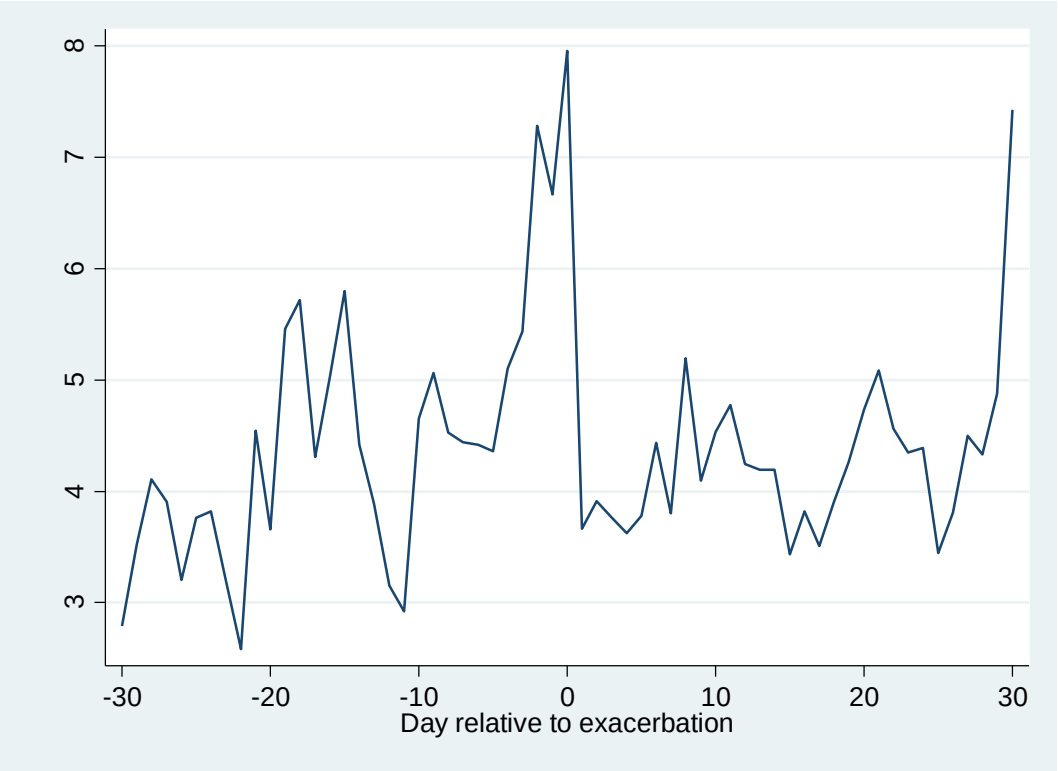
The final graphs are for reliever use, figure 14 is the graph from the fitted regression model and figure 15 uses the raw data for reliever use.

Figure 14 – Reliever use using regression model



The graph indicates that, as with the other outcomes, there is an increase in reliever use prior to exacerbation. However, for this outcome, the peak values are observed around a week before exacerbation. After this peak there is a decrease in scores up to exacerbation, and a further decrease post-exacerbation.

Figure 15 – Reliever use using raw data



5.0 DISCUSSION

5.0.1 Summary of key results

This study observed people with asthma and compared baseline, acute exacerbation and recovery states, examining biochemical signals to identify potential markers of asthma exacerbation.

Part 1

Part 1 of the study observed people with asthma who required hospital admission for acute asthma exacerbation. Admission (exacerbation) and the first follow up visit after 35 days in all participants, including fully recovered and recovering patients were compared. Statistically significant increases from admission to first follow up were observed in plasma PF4 (median 9 μg/ml (95 % CI 2, 17), p=0.002), eosinophils (median 0.13 $x10^{*}9/l$ (95% CI 0.10, 0.20), p=0.003) and eotaxin (median 9 pg/ml (95% CI 2, 17), p=0.002). Conversely, there was a statistically significant decrease from admission to follow up 1 in plasma IL-6 (median 0.0 pg/ml (95% CI -1.4, 0.0) p=0.02), IFN-γ (median -3 pg/ml (95% CI -10, 0) p=0.003), IL-13 (median 0.0 pg/ml (95% CI -0.2, 0.0) p<0.001), RANTES (median -601 pg/ml (95% CI -938, -266) p<0.001), IL-12 P40 (median 0 pg/ml (95% CI -30, 2) p=0.01), IL-17A (median -0.3 pg/ml (95% CI -3.2, 0.0) p=0.002), IL-5 (median -0.4 pg/ml (95% CI -1.5, 0.0) p=0.007) and urine GAG:creatinine (median -0.21 μg/mmol (95% CI -0.27, 0.03) p=0.04). Comparing admission to follow up 2 (mean 65 days) including those who had a delayed recovery and those who never recovered during the study, a statistically significant increase in plasma PAI-1 (median 6200 pg/ml (95% CI -1291, 10240) p=0.04) and eosinophils (median 0.10 $x10^{*}9/l$ (95% CI 0.00, 0.80) p=0.04) was observed.

There was no significant difference observed between asthma exacerbation and any follow up timepoint in Part 1 for plasma TAFI, D-dimer, fibrinogen, platelets, TGFβ1, VEGF, urine FDP or urine MMP activity.

Comparing exacerbation with recovery in all study subjects, a more clinically relevant comparison, there was a statistically significant increase in eosinophils (median $10^{*}9$/l (95% CI 0.10, 0.29) p=0.02) and eotaxin (median 12 pg/ml (95% CI 3, 22) p<0.001). Examining those who recovered rapidly at 35 days and comparing exacerbation with recovery, there was a statistically significant increase in eosinophils (median 0.10 $10^{*}9$/l (95% CI 0.07, 0.23) p=0.04) and eotaxin (median 12 pg/ml (95% CI 3, 22) p=0.002), and statistically significant decreases in IFN (median -3 pg/ml (95% CI -16, 0) p=0.01), IL-13 (median 0.0 pg/ml (95% CI -0.3, 0.0) p=0.01), RANTES (median -524 pg/ml (95% CI -1003, -45) p=0.03) and IL-5 (median -0.4 pg/ml (95% CI -2.0, 0.0) p=0.03). Examining eosinophilic and non-eosinophilic asthma subjects comparing exacerbation and recovery, there was a statistically significant increase in the eosinophilic group for eotaxin (median 6 pg/ml (95% CI 2, 24) p=0.02), and a statistically significant increase in the non-eosinophilic group for eosinophils (median 0.20 $10^{*}9$/l (95% CI 0.01, 0.20) p=0.03), TNFα (median 1.7 pg/ml (95% CI 0.1, 4.3) p=0.04) and eotaxin (median 17 pg/ml (95% CI 1, 27) p=0.01).

There were significant positive correlations at admission (exacerbation) between eosinophils and eotaxin (p<0.001), platelets and PF4 (p=0.02) and platelets and eosinophils (p=0.01).

Observing clinical parameters of exacerbation and recovery both PEFR (median 120 l/min (95% CI 87, 153) p<0.001) and FEV_1 (median 0.58 l (95% CI 0.43, 0.72) p<0.001) significantly increased at recovery compared with exacerbation. There was no statistically significant difference between exacerbation and recovery for FeNO.

Part 2

Part 2 prospectively observed participants at baseline, through exacerbation and to recovery. Looking prospectively at the relationship between a positive urine FDP result and asthma exacerbation, there was a statistically significant relationship. Examining the 8, 11 and 15 days prior to an exacerbation of asthma, the presence of a positive FDP result when compared with a negative FDP result was associated

with an odds ratio of 4.35 (95% CI 1.12, 16.9) (p=0.03), 5.50 (95% CI 1.38, 21.9) (p=0.02) and 6.07 (95% CI 1.43, 25.8) (p=0.02) respectively of a subsequent asthma exacerbation. This supports the initial hypothesis that markers of fibrin turnover are increased in blood and urine before and during exacerbation of asthma.

When comparing baseline state with asthma exacerbation, a statistically significant increase in plasma D-dimer (median 0.07 μgFEU/ml (95% CI -0.01, 0.15) p=0.02) and a significant decrease in plasma TGFβ1 (median -666 pg/ml (95% CI -1289, -2) p=0.02) was observed. Comparing baseline and recovery there was a statistically significant decrease in plasma PF4 (-1.7 μg/ml (95% CI -3.4, -0.1) p=0.04). There was no significant difference observed when comparing baseline to exacerbation or recovery in any of the other plasma markers examined. There was also a statistically significant increase in GAG:creatinine ratio in the 7 days preceding exacerbation (mean 0.45 μg/mmol (SD 0.18) p=0.03).

Clinically in Part 2, there were statistically significant increases in symptom score (p<0.001), reliever use (p=0.002) and composite score of symptom and reliever use (p<0.001) in the days preceding asthma exacerbation that fell during recovery. PEFR variability increased in the 7days (p=0.01) and 14 days (p=0.01) prior to exacerbation when compared with baseline state.

5.1 Plasma and urine parameters

5.1.1 FDP – urine

The primary objective of this study was to test the hypothesis that markers of fibrin turnover were detectable before and during asthma exacerbation in blood or urine. When looking prospectively, a significant increase in the incidence of asthma exacerbation following the detection of FDP in urine was observed. This was limited to the Part 2 population, and in part this is likely due to the more severe

asthma population when compared with the heterogeneity of Part 1, and possibly due to systemic treatment with corticosteroids. As discussed below, coagulation factors are consumed during fibrinogenesis and fibrinolysis results in the generation of FDPs (161). Although the test employed was a generic agglutination test for FDPs and could not specify which FDP was detected, a positive test correlated with an increased incidence of asthma exacerbation in the following 2 weeks of up to 6x that of a negative FDP result.

The strength of this study is not in the number of positive results but in the large number of negative results that were not followed by an exacerbation of asthma. Positive results were seen after exacerbation in the period of clinical recovery and before exacerbation without subsequent recovery. A return to a negative test after exacerbation is less useful in clinical terms as signs and symptoms are now present and their resolution can be utilised to monitor recovery. Whether subsequent exacerbation was more likely if further positive FDP results occurred was beyond the scope of this trial and in the one case where a second exacerbation occurred, the study participant was withdrawn from further study activity and results withdrawn from analysis.

The number of participants experiencing asthma exacerbation in this study makes sample size too small to calculate positive and negative predictive values of this test. Furthermore the test in its current form is neither sensitive nor specific enough to be used in clinical practice. However, with the supporting evidence of an increased plasma D-dimer in this cohort at the time of exacerbation, FDPs are a viable target for future study as a biomarker of asthma exacerbation. They have the potential to predict exacerbation prior to onset of clinical parameters enabling intervention and fulfil the required characteristics of a good biomarker, if a test can be developed prospectively that is sufficiently sensitive, specific and has accurate positive and negative prediction. Furthermore, the detection of signal in plasma and urine makes the possibility of translating such a marker into a point-of-care test more feasible.

In terms of the clinical potential to intervene a window of 7-14 days should be significant time to enable intervention. The FACET trial examined combined ICS/LABA inhaler use in asthma (162). This study identified an increase in symptoms 10 days before an asthma exacerbation that accelerated 5 days before (75). It is this window that has been successfully utilised in Symbicort Maintenance and Reliever Therapy (SMART) reducing time to severe exacerbation and number of those exacerbations (76). The optimal prophylactic or ameliorating regime would need to be established as would the optimal treatment window in prospective interventional studies.

5.1.2 Fibrinogenesis and fibrinolysis – plasma markers

This study measured markers of fibrin turnover in plasma of patients before, during and at recovery from acute exacerbation of asthma. Past studies have demonstrated that the balance of fibrin turnover is tipped in favour of fibrinolysis in the healthy lung, a state matched in moderate asthma treated with inhaled steroids (137). In severe asthma, the balance favours fibrinogenesis regardless of treatment with steroids, and withdrawal of ICS in moderate asthma alters the airway milieu to a pro-coagulant environment (80, 137). It is likely that an excess of PAI-1 is responsible for the shift towards fibrinogenesis observed (138, 163). PAI-1 reduces fibrinolysis by inhibiting plasminogen activation, an effect which promotes airway remodelling via TGF-β that is counteracted by uPA administration in murine studies (126). TAFI is also an inhibitor of fibrinolysis, however, in murine models of asthma, the presence of TAFI reduces AHR and airway inflammation, and reduced TAFI levels promote airway remodelling (139). This implies a mechanism of action for PAI-1 on airway remodelling and AHR beyond its direct effect on plasminogen activation. In light of these findings, it was expected that during acute asthma exacerbation there would be an increase in PAI-1 and a decrease in TAFI. In Part 1 of this study there was a significant increase in PAI-1 from exacerbation to follow up 2 (65 days) when looking both at those who recovered at follow up 2 and those who did not recover during the study period. When comparing exacerbation and all

subjects at first follow up (35 days), exacerbation and clinical recovery for all subjects, and exacerbation and delayed recovery there was also an increase at follow up visit but these results were not statistically significant. This trend might be explained by the proposed increased PAI-1 expression being localised to the airway rather than systemically in this setting. The trend for TAFI in all groups was for lower levels at exacerbation than recovery but again these results did not meet statistical significance. If significant this would be in line with expected trends. Prospectively, PAI-1 increased from baseline to exacerbation and fell again at recovery, and TAFI mirrored this effect falling during exacerbation and rising upon recovery but again, these trends did not meet statistical significance. It is likely that the Part 1 population was too heterogeneous to provide conclusive results whereas Part 2 was too small despite the more severe nature of these patients.

Fibrinogen is cleaved to fibrin during fibrinogenesis and therefore consumed, and one might expect reduced fibrinogen detected during asthma exacerbation. In both study arms no discernible difference was observed between any timepoints. This would be in keeping with the observation by Perrio and colleagues that TF-dependent coagulation of damaged airway epithelium occurs independently of plasma (92). If the components of the coagulation cascade are produced and stored locally in airway epithelium, then a difference systemically may not be perceptible in coagulation factors between exacerbation and stable states. Furthermore, coagulation factors are consumed during fibrinogenesis and with the exception of FDPs, nothing remains after fibrinolysis.

A difference was observed between baseline and exacerbation of D-dimer, but only in the Part 2 population. D-dimer is a breakdown product of successful coagulation and indicates that both fibrinogenesis and fibrinolysis has occurred. D-dimer is detectable systemically in a wide range of conditions, most widely recognised and utilised in the setting of venous thromboembolism (VTE) (161). The observed difference in Part 2 and not Part 1 is again a likely reflection of the asthma severity

in the Part 2 population compared with the heterogeneity of Part 1 subjects (137). This also reflects the pro-coagulant state observed by Majoor and colleagues who observed increased VTE in severe asthma and those requiring oral corticosteroids (164). The fact that only D-dimer was detectably increased at exacerbation of all the markers of fibrin turnover examined could be explained by the consumption of other coagulation factors during fibrinogenesis and subsequent fibrinolysis.

5.1.3 Platelets and PF4

In Part 1, PF4 was observed to be lower at exacerbation than first follow up at 35 days, with no change in platelet counts between timepoints. As discussed in chapter 2, laboratory analysis was not performed at follow up in a large number of study subjects reducing the power of looking at platelet numbers directly. Furthermore, the method used to prepare plasma in this study did not remove platelets completely from plasma samples. However, standardised methods of platelet preparation were used, which should result in similar platelet concentrations in each sample. Any excess platelet contents released by cell lysis during freeze-thaw cycles should be uniform. PF4 is contained in platelet alpha granules and is released on platelet activation (81), so any detectable difference between PF4 levels should represent peripheral platelet activation, or a surrogate marker of peripheral blood platelet count. Indeed a positive correlation was observed between platelets and PF4 at admission. This was not observed at subsequent timepoints most likely due to the reduced specimen numbers. Although differences between samples is likely to reflect differences in platelet activation and platelet count, the absolute values of factors contained in platelets are likely to be higher with these plasma samples than studies using platelet-poor or platelet-deplete plasma. This should be noted if comparing this study with other published work. Tutluoglu and colleagues observed higher peripheral blood PF4 levels in asthma exacerbation compared with healthy controls (136), and PF4 increased after treatment of exacerbation with oral steroids. In a murine model of asthma, allergen challenge induced platelet migration into lung tissue (165),

furthermore platelets expressing P-selectin may be required for successful translocation of eosinophils into the lung (166). In the present study, both platelets and eosinophils have been observed at lower levels in peripheral blood during exacerbation than at recovery, a finding that is consistent with such interdependent sequestration into the lung. Furthermore, platelet and eosinophil counts demonstrated a significant correlation at admission supporting this hypothesis. Again, no correlation was seen at follow up visits likely due to specimen numbers as highlighted above.

The treatment of this population with oral steroids and subsequent increase in PF4 during recovery would be consistent with Tutluoglu's observations (136) which could be explained by platelets that acutely migrated to the lungs during exacerbation being returned to the systemic circulation on recovery, perhaps because of the treatment with steroids. In the prospective study arm, there was no significant change between baseline and exacerbation or between exacerbation and recovery, however a significant fall in PF4 was observed when comparing baseline and recovery. It is likely that this is due to the effect of steroids described above and the lack of signal between other timepoints is probably explained by overall small sample size.

5.1.4 Eosinophils

In Part 1 of this study, lower levels of peripheral eosinophils were observed at exacerbation compared with recovery. Airway eosinophilia is associated with exacerbation of asthma (167) and reduction of sputum eosinophils reduces asthma exacerbation (32). The observed reduction in peripheral eosinophils during exacerbation could be explained in two ways. Firstly, eosinophils migrate into the airway lumen in response to acute inflammatory insult (168) such as that seen during acute exacerbation of asthma. A reduction in peripheral eosinophil counts may be due to eosinophil migration into the airways in response to inflammation

and chemotaxis. Secondly, many of the study subjects received oral steroids prior to plasma samples being collected. Although attempts were made to record the interval from first steroid dose to sample collection accurately, this was not consistently achieved for methodological reasons discussed in Chapter 2. Although not accurate enough for formal statistical comparison, it is clear that considerable variation in this interval occurred, some subjects receiving oral steroids several days prior to admission, and in others this interval was only minutes. The effects of treatment with oral steroids are rapid, with peak plasma levels being achieved in under an hour (169), and clinical effects being seen within 3 hours (4). Although efforts were taken to assess patients for study participation as soon as possible after admission, delaying treatment for study participation was outwith the study protocol and was not practiced, therefore treatment effects may have influenced the observed peripheral eosinophil counts. It is worth noting that although statistically significant, the fall in peripheral eosinophils would not normally be classed as significant in terms of clinical practice, in fact median peripheral eosinophil counts at exacerbation and recovery were within the normal range for adult subjects (see appendix 20). A significant correlation between eosinophil count and eotaxin was observed at admission which is expected as eotaxin is an eosinophil chemokine. This correlation was not observed at subsequent timepoints but as discussed in chapter 2, blood samples for FBC were not routinely taken at follow up visits reducing the power of these data for analysis at follow up visits.

5.1.5 VEGF

VEGF is a potent angiogenic factor that is present at higher levels in the sputum of asthmatic individuals when compared with healthy controls (170). The effects of VEGF in asthma include increased angiogenesis and vascular leakage, but also increased eosinophilic inflammation, AHR and airway remodelling demonstrated by subepithelial collagen deposition and smooth muscle hyperplasia in murine models (171). When comparing asthmatic subjects with healthy controls, Lee and colleagues found increased levels of VEGF in plasma in asthmatic subjects that

increased during exacerbation and subsequently fell with treatment over a 28 day period (74). This could not be replicated in either study arm despite a variety of methodological considerations discussed in chapter 2. Inhaled corticosteroids in different formulations and models have been shown to affect VEGF and its effects in asthma. Inhaled budesonide in a murine model inhibited lung VEGF levels and angiogenesis (172). Higher sputum VEGF has been observed in steroid-naïve asthmatic subjects compared with healthy controls, with VEGF levels negatively correlating with FEV_1, and a subsequent reduction in sputum VEGF after inhaled BDP (173). Similar observations have been made with inhaled fluticasone propionate in terms of reduced VEGF angiogenesis (174), and with BDP reducing VEGF and exercise-induced bronchospasm (175). Combined therapy with budesonide/formoterol over 6 months reduces epithelial VEGF and VEGF receptor levels (176). The failure to replicate the findings of Lee and colleagues in this study, is likely due to the population being "real life" asthmatics already receiving treatment with inhaled therapies, whereas Lee's study population had not received inhaled or oral steroids for at least 6 months prior to study recruitment (74).

In terms of the wider interactions of VEGF, it is important to note that TGFβ1 stimulates VEGF expression *in vitro* (177). Furthermore, *in vitro* stimulation of human lung epithelial cells by VEGF increases TGFβ1 and inhibition of VEGF reduces TGFβ1, an observation repeated in a murine model (178). Examining the relationship between VEGF and MMP, in a murine model of asthma MMP-9 and VEGF levels are increased, correlate with each other, and the inhibition of VEGF reduces MMP-9 levels (73).

5.1.6 TGFβ1

TGFβ1 has both pro- and anti-inflammatory properties. It inhibits Th1 and Th2 responses reducing the inflammatory response, yet it is a potent chemotactic factor and activator that is released from inflammatory cells such as eosinophils and

macrophages, alongside structural cells including fibroblasts, epithelial cells and smooth muscle cells (179). In asthma, eosinophils are the main source of TGFβ1 and it is postulated that an initial lack of TGFβ1 leads to a lack of T regulatory cells allowing Th2 airway inflammation, eosinophilic inflammation and a resultant increase in TGFβ1 and subsequent airway remodelling (179). TGFβ1 has potent effects on airway remodelling via epithelial mesenchymal transition (EMT) both *in vitro* (180) and *in vivo* (69). This EMT effect is greater in asthmatic epithelium than in healthy controls (69) and *in vitro*, there is a synergistic effect on EMT between TGFβ1, IL-4 and IL-17 (Th2 and Th17 cytokines respectively) (181). This is supported by the observation that TGFβ1 alone requires prolonged exposure to airway smooth muscle cells to induce proliferation (182). The administration of anti-IL-5 reduces airway eosinophils and also airway remodelling by removing the main source of airway TGFβ1 (183). However, the dual role of TGFβ1 is illustrated by the fact that in a murine model of asthma, suppression of TGFβ1 reduced airway fibrosis at the expense of increased AHR (184).

In Part 1 of the study no difference was observed in plasma TGFβ1 between acute exacerbation and any follow up time point. Plasma TGFβ1 is higher in stable non-atopic asthmatics compared with atopic asthmatics or healthy controls (185). Serum TGFβ1 has also been observed at higher levels in patients with asthma than healthy controls but higher still in steroid naïve asthma compared with moderate treated or remission asthma populations (186). This is supported by an observed correlation between asthma control measured using the asthma control test (ACT), where uncontrolled asthma was associated with higher plasma TGFβ1 (70). Higher BALF levels of TGFβ1 are seen in asthma than in healthy controls and these levels rise with allergen challenge (187). This would be more analogous to acute exacerbation than steroid naivety or poor asthma control, however, it may be that such acute reactions are confined to the lungs hence why they could not be measured in plasma in the present study. Indeed, in Part 2 a significant fall from baseline to exacerbation in plasma TGFβ1 was observed, which may represent a

translocation of TGFβ1 into the lungs possibly via inflammatory cell migration, although a significant fall in eosinophil numbers from baseline to exacerbation was not seen. The population for Part 2 were all in the moderate to severe asthma groups whereas Part 1 included milder severities of asthma which may have influenced these results. Finally, inhaled therapy with budesonide/formoterol has been shown to reduce TGFβ1 expression and spirometric and computed tomographic markers of airway remodelling (188). The study populations were all taking inhaled therapy prior to study recruitment which may have suppressed any detectable difference in TGFβ1.

5.1.7 MMP

The matrix metalloproteinases (MMPs) are key enzymes involved in ECM breakdown and airway remodelling (189). MMP-9 is particularly relevant and the balance between MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) is increasingly recognised as important in airway remodelling in asthma (190). We could not detect a significant difference in urinary MMP activity between exacerbation and recovery states. This is contrary to the findings of Mak and colleagues who observed higher plasma MMP-9 during asthma exacerbation that reduced on recovery but remained higher than healthy controls (191). One explanation might be our attempt to detect MMP in urine rather than plasma, however, MMPs have been detected due to other conditions not directly affecting the kidney or urinary tract (192). In our study, analysis of MMP was limited to Part 1 subjects, a more heterogeneous population in terms of asthma severity. Exhaled MMP-9 levels are higher in severe asthma than in mild to moderate asthma (193) and differential MMP expression and activity is observed in different asthma inflammatory phenotypes (194). This may account for our failure to observe a difference between exacerbation and recovery in the Part 1 population. Glucocorticosteroids and long-acting β_2 agonists attenuate MMP-9 expression from human bronchial epithelial cells (195). The majority of our population were receiving inhaled therapy with 1 or both of these drugs and a significant proportion

had received high dose nebulised β_2 agonists and oral corticosteroids prior to urine collection. This could well confound our ability to detect any significant difference.

5.1.8 GAGs

The glycosaminoglycans are involved in airway remodelling and inflammation in asthma. In controlled asthma, circulating endogenous heparin is lower than healthy controls (65), possibly contributing to increased inflammation in asthma. Attempts to use heparin as a therapeutic agent in asthma have been made for many years with variable success (196, 197). In both human (63) and murine studies (198), GAG deposition in asthmatic airways has been observed. The effects of inhaled corticosteroids and LABA include counteracting the pathologic degradation of hyaluronic acid, reducing the pro-inflammatory potential of airway smooth muscle cells (199). This is in line with the observation by Priftis and colleagues that urinary GAG excretion is increased in children with asthma taking ICS, but not those taking reliever medication alone (200). Presumably this effect is due to the anti-inflammatory effects of steroids and reduction in bronchial hyperreactivity reducing airway deposition of GAGs and subsequent remodelling. Our finding that urine GAG excretion is increased during asthma exacerbation and reduces at 35 day follow up is in keeping with the observation of Shute and colleagues (201), who observed increased GAGs in urine during acute severe asthma and a return to normal levels over 28 day follow up.

5.1.9 Cytokines and chemokines

A multiplex panel of chemokines, cytokines and pro-inflammatory mediators was examined to explore the detectable inflammatory changes in the study population. The intention in performing this multiplex analysis was to aid characterisation of inflammatory phenotype in this cohort in response to the lack of sputum data obtained. The results demonstrate a systemic inflammatory response, not limited to an individual phenotype. When measured peripherally, eotaxin was observed to

be lower at exacerbation. This could represent cellular migration to the airways and had sputum been successfully measured, a direct comparison could have been made. This observation might also represent an effect of acute treatment with oral corticosteroids. A relatively strong correlation between eosinophil count and eotaxin at admission was observed which would support this hypothesis.

In Part 1, higher levels of IL-6, IFN-γ, IL-13, RANTES, IL-12P40, IL-17A and IL-5 were observed at exacerbation compared with follow up (mean 35 days), eotaxin was lower during exacerbation than recovery, and no difference was observed in IL-8 or TNF-α between timepoints. Comparing exacerbation with clinical recovery, a statistically significant fall was observed in IFN-γ, IL-13, RANTES and IL-5, with a significant rise in eotaxin. The subjects in this study are their own controls, and these observations imply a rise in plasma cytokine levels during exacerbation that returns to baseline at recovery, except eotaxin, which falls during exacerbation and rises on recovery.

These findings demonstrate a systemic increase in Th1 cytokines (IFN-γ, IL-12P40), Th2 inflammatory (IL-5, IL-13) and regulatory (IL-17A, IL12P40) cytokines, pro-inflammatory cytokines (IL-6), T cell/eosinophil chemokines (RANTES) and a decrease in the eosinophil chemokine Eotaxin. These findings are consistent with the observations of other investigators. Lee and colleagues observed increased serum IL-5, IL-13 and a trend towards increased IFN-γ during acute asthma exacerbation (202). Zietkowski and colleagues observed increased RANTES in the airway during acute asthma exacerbation (203). Kaminska observed IL-12, IL-13 and IFN-γ in bronchial biopsies from asthmatic airways with fixed airflow obstruction and IL-17 and RANTES in those with intermittent obstruction (204). Kato observed increases in serum IL-5 and IL-6 during acute asthma exacerbation (205). Elevations in sputum levels of Eotaxin have been observed during acute exacerbation of asthma (206).

In Part 2 of the study, no difference was observed in any marker comparing baseline with exacerbation or recovery. It is likely that the small sample size limited the detection of any significant differences in this study arm.

The observed differences in cytokines and chemokines between different clinical scenarios in asthma raise interesting questions and possible hypotheses. It is important, however, to acknowledge that the intention of these analyses was to characterise disease phenotype, an objective that was unsuccessful, and no correction was made for multiple comparisons potential weakening the validity of the observed differences (207). These parameters are not measured routinely in clinical practice and hence a normal range is not established to give reference to the measured values. It is clear however, that while some of the measured changes may be statistically significant, any clinical significance must be questioned, for example the average decrease observed in IL-13 from 0.0 to 0.0 (table 5).

5.2 Clinical parameters

This study recruited patients with asthma who experienced an acute exacerbation requiring treatment with oral corticosteroids, and their treatment was provided by clinical teams according to standard practice and national guidelines (4). The data collected has highlighted clinical observations that merit further discussion and prompt future research questions and important consideration for the design of subsequent studies.

5.2.1 Asthma exacerbation

The NHLBI define an asthma exacerbation as an "acute or subacute episode of progressively worsening shortness of breath, cough, wheezing, and chest tightness – or some combination of these symptoms. Exacerbations are characterised by decreases in expiratory airflow that can be documented and quantified by simple measurement of lung function" (1). GINA definitions are similar in description and

equally lacking in quantifiable measures defining presence or absence of exacerbation (5). The BTS delineates the severity of an exacerbation into moderate, acute severe and life threatening based on a series of clinical parameters measured once exacerbation is diagnosed (4). None of these definitions gives a clear objective definition that can be agreed on for the purposes of research, or for consistency in clinical practice, leaving a large part to the subjective experience of the patient combined with individual judgement of the treating clinician. The ATS/ERS statement on asthma research gives a similar description as those described above but clarifies a severe exacerbation in terms of research should require treatment with systemic corticosteroids or require hospital admission (6).

The present study defined exacerbation as requiring oral corticosteroids in line with a severe exacerbation. It is a strength of this study that the decision to treat and admit was left to the discretion of the treating clinician in line with national guidelines (4). Due to the rather nebulous definition outlined in national and international guidelines, it is an important point to consider when designing future studies how objective one can be when diagnosing asthma exacerbation.

5.2.2 Recovery from exacerbation

None of the national or international asthma guidelines define recovery from an exacerbation of asthma. The BTS advise improved symptoms and PEFR rather than a return to normality should be used to guide decisions on discharge from the emergency department (4), GINA advises PEFR may be useful to monitor recovery (5), and the NHLBI do not refer to recovery in their guideline (1). Recovery is not described by the ATS/ERS joint statement on asthma research (6).

For the purposes of this study, the decision was taken to define recovery as a return to pre-exacerbation state defined subjectively by the patient. If asthma control was

poor prior to exacerbation and a return to this level of control was achieved, this was considered a recovery. The choice of 4 week initial follow up was based anecdotally on received wisdom that inflammatory changes occurring due to asthma exacerbation take approximately 6 weeks to return to normal, accepting symptoms may improve prior to this. On subsequent literature review, no reference could be found to a 6 week figure, in fact there is a paucity of any studies commenting on recovery after asthma exacerbation.

The consistency with real life practice is a strength of this study but once again, the definition of recovery from asthma exacerbation should be considered in future study design and is an area that merits further study.

5.2.3 FeNO during acute exacerbation

The use of sputum eosinophil counts to guide asthma management has been shown to reduce exacerbation frequency and reduce steroid doses used for treatment (32). The use of FeNO as a surrogate marker of sputum eosinophilia has been less convincing in terms of outcome data (48). However, recent ATS guidelines acknowledge the advantages and limitations to the test and recommend its use for the diagnosis of eosinophilic airway inflammation and assessing likelihood of steroid responsiveness (49). There has been limited study of FeNO during acute exacerbation. One ED study was discontinued as reproducibility of FeNO measurement acutely was considered unacceptable (208) a second paediatric study observed elevated levels of FeNO during asthma exacerbation, however no cut off level for exacerbation diagnosis could be established (209).

FeNO was measured in Part 1 subjects at exacerbation and again on recovery. No significant difference was observed in measured FeNO at exacerbation compared with recovery. Even when this comparison was limited to those study participants

with an eosinophilic inflammatory phenotype, no difference in FeNO was observed between exacerbation and recovery. While it is recognised that in this study, the total population who recovered with data recorded at both timepoints for comparison was small (54 subjects), and that the eosinophilic subgroup was smaller still (30 subjects), this remains one of the biggest cohorts to date examining FeNO during acute exacerbation of asthma and again at recovery.

5.2.4 Eosinophilic phenotype

As discussed in Chapter 2, sputum sampling was not successful in the majority of study participants. The decision was taken, therefore, to categorise eosinophilic phenotype using the method described by Schleich and colleagues (160) utilising one or more of a raised peripheral eosinophil count, raised IgE or raised FeNO. Where sputum was obtained, a differential sputum eosinophil count of >3% was deemed significant. If these criteria were met at any study timepoint, the subject was deemed to have an eosinophilic inflammatory phenotype.

It is worthy of note that eosinophilic inflammation changed between timepoints for study subjects in no particular pattern. Some had eosinophilia present through study visits, some present only at exacerbation and some only at recovery. Phenotypic lability such as this has been observed in children with asthma (210) and is unrelated to changes in steroid treatment. This observation highlights the importance of careful characterisation of an individual's asthma phenotype using all available information. In terms of future study design and using phenotypes to guide therapy, there may be a need to examine parameters such as sputum, peripheral blood and FeNO in a longitudinal manner before an individual's phenotype is determined.

5.2.5 Objective markers of recovery and exacerbation

As discussed above the definition of asthma exacerbation has been described by guidelines and parameters are described that determine severity of exacerbation (4). These require an individual to be deemed as having an asthma exacerbation to apply and in terms of research, it is important to try and define an exacerbation by clearly determined parameters. This argument may seem pedantic, however, when attempting to confirm an asthma exacerbation in this study, it was determined that an exacerbation was based on a treating physician deeming it was occurring, due to an absence of accepted measurable parameters. Those parameters that are described such as PEFR require knowledge of PEFR when well for comparison, a fact not known by a significant proportion of this population, or comparison with a predicted value, often different from best in those who did know. Similar difficulties arose when defining recovery from exacerbation.

In Part 1 of this study, a significant rise was observed in both FEV_1 (mean 600 mls) and PEFR (mean 120 l/min) from exacerbation to recovery. This provides objective measurement of improvement in airflow obstruction at recovery but is limited by a lack of baseline data to demonstrate a fall during exacerbation.

Study participants were also asked to record the presence or absence of common asthma symptoms on a daily basis and the number of times a reliever was used, again in an attempt to provide a measurable distinction between exacerbation and recovery states. A symptom score from 0-4 was derived from the presence or absence of wheeze, night wakening, chest tightness and breathlessness during a 24 hour period. Reliever use, either number of puffs of SABA and/or number of SABA nebulisers in 24 hours was also recorded and assigned a numerical value as outlined in table 32. The composite score from 0-7 combines symptom score and reliever use.

Table 32 – Symptom and composite scores	
Parameter	**Score**
Wheeze	1
Night waking	1
Chest tightness	1
Breathlessness	1
Reliever use	
≤ 2	0
3 – 9	1
≥ 10	2

This scoring system gives a score between zero and seven on any one day. Other scoring systems have been described and validated for evaluating asthma control (75, 211), however the intention in our study was not to look at asthma control, rather to provide objective evidence of exacerbation. It was intended that the simplicity of binary questions, i.e. presence or absence of each symptom, would remove some of the subjectivity present in numerical scales used in other scores.

In the prospective Part 2 data, statistically significant rises were observed in symptom score and reliever use independently, and combined as a composite score. When examined alone, reliever use was positively skewed requiring logarithmic data to be evaluated. This makes it less practical as a tool to be used in clinical or research practice. However, the symptom and composite scores are simple to record and have provided objective evidence of exacerbation. Consideration should be given to prospective evaluation of these scores in larger cohorts to validate their use. They could be of particular benefit for future research studies to help define exacerbation and subsequent recovery. The temporal relationship observed is that symptoms climb in the 10 days preceding exacerbation and peak just after, whereas reliever use begins to rise 7 days prior to exacerbation and peaks just before. Both parameters fall after exacerbation.

The prospective PEFR data did not demonstrate a significant fall in mean PEFR prior to exacerbation, however there was a statistically significant fall in PEFR variability when compared with baseline. Clinically the fall was only 20 l/min, a mean 7% fall for our population classed as below the threshold of normal variability for PEFR measurement and not regarded as a significant fall from baseline in terms of asthma exacerbation (4). This contradicts the findings of Tattersfield and colleagues who observed falls in PEFR prior to exacerbation of up to 20% in the FACET study (75). However, the present study examined 23 subjects, only 15 of whom had adequate PEFR data compared with 425 subjects in the FACET study, 114 of whom were diagnosed as exacerbating by a fall in PEF of 30% or more from baseline. Furthermore, the FACET trial examined mean morning and mean evening PEFR values where the present trial examined PEFR variability. This could explain the smaller "sub-clinical" fall in PEFR for the present population.

Symptom data from the FACET trial shows a rise in symptoms in the 10 days preceding exacerbation (75). The present study demonstrated comparable significant increases in symptom score in the 10 days prior to exacerbation. The symptom score used in the present study has the added benefit of simplicity in terms of data recording. Other studies suggest a symptom diary may be a more sensitive tool for revealing acute exacerbation of asthma than measures such as PEFR (212). It is worthy of note that the FACET trial found that for increased PEFR variability, increased symptoms or increased reliever use, the odds ratio of subsequent exacerbation was statistically significant, but did not exceed 1.1 for any of the variables. In comparison, an odds ratio of between 4 and 6 for subsequent exacerbation following a positive urine FDP warrants further study.

5.2.6 Who recovers quickly?

Initial analysis looking at inflammatory phenotype defined by sputum differential cell count suggested that eosinophilic asthmatics in this cohort were more likely to

recover rapidly than neutrophilic asthmatics. As discussed, the limited sputum data made such comparisons too small in terms of sample numbers to draw any firm conclusions. Subsequent analysis of this cohort looking only at those who recovered and looking at the relationship between rate of recovery and presence of eosinophilic inflammation did not show any significant correlation.

5.3 Conclusions

This is the largest study to date examining coagulation and fibrinolysis in acute exacerbation of asthma. In individuals with moderate to severe asthma, urine FDPs were detected in the days preceding an asthma exacerbation, prior to patients developing symptoms sufficient to seek medical attention. The detection of FDP in urine was associated with a significantly increased risk between 4 and 6 times more likely of subsequent asthma exacerbation in the following 7 to 14 days when compared with a negative urine FDP test. Fibrin(ogen) degradation products are promising urinary biomarkers of asthma exacerbation that warrant future study and detecting FDPs in urine presents the realistic prospect of translating these findings into a practical and acceptable point-of-care test for patients with asthma. This is supported by the observation that prospectively D-dimer, a fibrin degradation product, was significantly increased in plasma at exacerbation compared with baseline.

The present study examined plasma markers of fibrin turnover alongside cytokines, chemokines and blood cells. Although various parameters significantly decreased from exacerbation to follow up, when comparing exacerbation and clinical recovery, no markers of fibrin turnover significantly changed in plasma. A significant increase in eosinophils, eotaxin and PF4, alongside observed positive correlations at exacerbation between these parameters and platelets, supports the migration of eosinophils and platelets into the lung during asthma exacerbation. Future study of coagulation and fibrinolysis in asthma may benefit from focussing on airway samples in preference to plasma for this reason.

Clinically, this study demonstrates that FeNO is not useful for distinguishing asthma exacerbation from baseline or recovery states. The symptom and composite scores used in this study provide objective evidence of asthma exacerbation for use in future research studies and would benefit from prospective evaluation and validation in future larger cohorts.

6.0 BIBLIOGRAPHY

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7.0 APPENDICES

Appendix 1 – Beclometasone dipropionate (BDP) equivalent doses of inhaled corticosteroids

Equivalent doses of inhaled corticosteroids adapted from BTS asthma guideline 2011	
Steroid	**Equivalent dose**
Beclometasone	
Clenil modulite	400mcg
Clickhaler	
Aerobec Autohaler	
Asmabec Clickhaler	
Dry powder (becodisks)	
Easyhaler	
Pulvinal	
Filair	
Qvar	200-300mcg
Fostair	200mcg
Budesonide	
Turbohaler	400mcg
Metered dose inhaler (MDI)	
Easyhaler	
Novolizer	
Symbicort	
Symbicort (regular and as required dosing)	
Fluticasone	
MDI	200mcg
Accuhaler	
Seretide MDI	
Seretide Accuhaler	
Mometasone	200mcg
Ciclesonide	200-300mcg

Appendix 2 – GINA classification of asthma severity before treatment 2008

GINA classification of asthma severity by clinical features before treatment (worst feature determines severity)	
Intermittent	
Symptoms < once a week	
Brief exacerbations	
Nocturnal symptoms not > twice a month	
FEV_1 or PEFR ≥ 80% predicted	
PEFR or FEV_1 variability <20%	
Mild Persistent	
Symptoms > once a week but < once a day	
Exacerbations may affect activity and sleep	
Nocturnal symptoms > twice a month	
FEV_1 or PEFR ≥ 80% predicted	
PEFR or FEV_1 variability <20-30%	
Moderate Persistent	
Symptoms daily	
Exacerbations may affect activity and sleep	
Nocturnal symptoms more than once a week	
Daily use of inhaled SABA	
FEV_1 or PEFR 60-80% predicted	
PEFR or FEV_1 variability >30%	
Severe Persistent	
Symptoms daily	
Frequent exacerbations	
Frequent nocturnal asthma symptoms	
Limitation of physical activities	
FEV_1 or PEFR ≤ 60% predicted	
PEFR or FEV_1 variability > 30 %	

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Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

Study Protocol Version 1.4
01/03/2011

REC no: 10/H0505/59

Chief Investigator: Prof Anoop J Chauhan

Principal Investigator: Dr Jonathan J Owen

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

HYPOTHESIS

Acute exacerbation of moderate and severe asthma is associated with an increase in fibrin formation in the airways and that fibrin degradation products in sputum, blood and/or urine are increased compared to stable disease. Furthermore, urinary measurements in the week before may predict the onset of an exacerbation and allow monitoring of response to therapy.

BACKGROUND

Inflammation

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. In susceptible individuals, this inflammation causes recurrent episodes of coughing, wheezing, breathlessness, and chest tightness. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. [1] Asthma can cause irreversible airflow obstruction and the degree of obstruction is a function of the duration and severity of previous asthma. [2] Severe asthma exacerbations requiring emergency treatment or hospital admission are associated with a more rapid decline in lung function. [3] Airway remodelling occurs in asthma and is characterised by goblet cell hyperplasia and metaplasia, an increase in bronchial smooth muscles and blood vessels, and deposition of interstitial collagens that extends beyond the thickened lamina reticularis to involve the entire airway wall in proportion to disease severity. [4] Fibroblast differentiation to myofibroblasts occurs as part of this remodelling process. [5]

Epithelium

The airway epithelium acts as a defensive barrier to injury from inhaled particles, infectious agents and allergens. Bulk exudation of plasma proteins can occur across an intact epithelial membrane which is activated by inflammatory mediators but not necessarily damaged, implying a significant role in mucosal immune defence, inflammation and repair. [6] However, the epithelium in asthma is fragile becoming chronically injured and unable to repair itself properly. The permeability of asthmatic epithelium is greatly increased, leading to greater access of inhaled allergens, pollutants and other irritants to the underlying airway tissue. [5] Damage to and shedding of airway surface epithelium are often noted in studies of asthma and the greater the loss of surface epithelium in biopsy specimens, the greater the degree of airway hyperresponsiveness. [7] Injury to the airway epithelium is followed by rapid migration of epithelial cells to cover the denuded area. These cells then proliferate and differentiate followed by apoptosis to remove excess numbers of epithelia and restore the normal proportions of cell types. Epithelial cell adaptation can occur to allow faster repair if recurrent exposure takes place. [8] Within minutes of epithelial injury, plasma exudation occurs with the formation of a gel-like network of fibrin and other plasma proteins on the denuded basement membrane. [9]

Haemostasis

The coagulation cascade is a series of proteolytic reactions which occur in response to endothelial injury and result in the formation of a fibrin clot (fibrinogenesis). The extrinsic cascade is the main initiator of the coagulation pathway and is activated by the exposure of subendothelial cells to plasma. Tissue factor (TF) expressed on subepithelial cells reacts with activated factor VII (FVIIa) to form the TF-FVIIa complex which cleaves FX and FIX to their

activated forms (FXa and FIXa respectively). FXa then cleaves prothrombin to thrombin initially in a small amount which acts as a primer to activate FV, FVIII, FXI, FXIII and platelets. FXIa cleaves further FIX which complexes on the activated platelet surface with FVIIIa to cleave further FX. The resulting FXa forms a complex with FVa on the platelet surface and cleaves prothrombin to thrombin in the large amounts needed to cleave fibrinogen to fibrin. [10]

The fibrinolysis pathway removes the fibrin clot once epithelial repair has occurred to restore vessel patency. Tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) cleave plasminogen to plasmin which cleaves fibrin forming fibrin degradation products including D-dimer, which consists of 2 fibrin monomers cross-linked by FXIIIa. [10]

Regulation of these pathways occurs by protease inhibition or proteolytic degradation via negative feedback and activation of specific inhibitors to pathway components. Thrombomodulin expressed on intact endothelial cells surrounding an area of damage bind thrombin and activate protein C (APC) which inactivates FVIIIa and FVa. Antithrombin (AT) inactivates thrombin, and tissue factor pathway inhibitor (TFPI) inactivates the TF-FVIIa-FXa complex. The action of AT and TFPI are augmented by the anticoagulant heparin. tPA is inactivated by plasminogen activator inhibitors (PAI) and plasmin is inactivated by thrombin-activatable fibrinolysis inhibitor (TAFI). [10]

The roles of coagulation and fibrinolysis in asthma

Dysregulation of the balance between fibrinogenesis and fibrinolysis has been recognised in acute lung injury (ALI), adult respiratory distress syndrome (ARDS), systemic sclerosis, idiopathic pulmonary fibrosis, sarcoidosis, cryptogenic organising pneumonia and interstitial lung diseases associated with collagen vascular disease. [11] The role of these pathways in the pathogenesis of asthma is becoming the focus of increasing study.

Fibrin is thought to provide a provisional matrix on which fibroblasts proliferate and produce collagen [12], implying a possible role in the airway remodelling that occurs in asthma. Furthermore, reduced surfactant activity is observed in asthma and inhalation of surfactant reduces airway hyperresponsiveness (AHR). The most powerful protein inactivator of surfactant, to the best of our knowledge, is fibrin. [13] Increased fibrin deposits have been observed post mortem in a patient, who died of status asthmaticus, an effect replicated in mouse models of allergic airways inflammation, accompanied by suppression of fibrinolysis as illustrated by increased PAI activity and reduced plasminogen activity. In the same model increased AHR was induced by nebulised fibrinogen followed by thrombin and reduced by nebulised tPA, implicating fibrin as a potential mediator of AHR. [13]

TF is constitutively expressed on bronchial epithelial cells and inhibition of TF activity significantly inhibits bronchial epithelial wound repair. [14] Increased TF is found in the sputum of asthmatics compared with healthy controls and this correlates with increased levels of thrombin. [15] Eosinophils play a major role in the inflammation and AHR of asthma. Eosinophils contain significant intracellular stores of preformed TF, and once activated, they express TF on the cell surface which regulates migration across epithelial layers. [16] TF is also expressed on human fibroblasts, [17] which play a role in asthmatic airway remodelling.

Antigen challenge in asthmatic airways causes an increase in thrombin activity alongside increased inflammatory cells in broncho-alveolar lavage fluid (BALF). This fluid can induce bronchial fibroblast proliferation and is inhibited by hirudin, a specific thrombin inhibitor. [18] Suppression of FXa with fondaparinux has been shown in a murine model to attenuate AHR and reduce airway wall thickness. [19] The compressive stress on bronchial epithelial cells, such as occurs in bronchoconstriction in asthma, increases expression of uPA, PAI-1, tPA and matrix metalloproteinases (MMPs). [20] PAI-1 has been linked with airway remodelling and uPA with cellular inflammation in asthma. [21] FXa has also been implicated in tissue

remodelling and inflammation through proteinase-activated receptor (PAR) cell signalling. [22] Plasmin also plays a role in airway remodelling directly and via the activation of MMPs. [23]

Coagulation factors in Moderate and Severe Asthma

In our studies of subjects with *moderate* asthma, the fibrinolytic environment may activate matrix metalloproteinases promoting destruction of the extra-cellular matrix (ECM) via enhanced plasmin production (e.g. through increased tPA and urokinase plasminogen activator (uPA) release or inhibition of PAI-1). [24] This fibrinolytic environment was reversed by inhaled corticosteroids, and this would encourage epithelial repair by improving the stability of newly formed fibrin matrices. [25] It is possible that inappropriate activation of this plasmin system would accelerate the production of inflammatory mediators and growth factors that influence airway remodelling. [23]

In subjects with *severe* asthma, procoagulant factors contributing to airway remodelling, persisted at elevated levels despite corticosteroid therapy, and were independent of inflammatory cell influx. Thus analysis of a2-macroglobulin levels (surrogate of plasma exudation) in sputum established that TF, tPA and PAI-1 were present in sputum at levels much higher than could be accounted for by plasma exudation, and were likely produced by local cellular sources (e.g., epithelium or fibroblasts) rather than from circulating cells. [25]

Coagulation Factors during Exacerbations

Viruses (the commonest triggers for asthma exacerbations) could thus also affect this airway milieu. Increased expression of VEGF following viral infection of bronchial epithelial cells [26] is likely to be an important early event, leading to vascular permeability, and inflammation. [27] Furthermore, IL-13 is linked to fibrin formation in asthmatic subjects as it induces both VEGF and TGFb [28] following experimental viral infection. Airway obstruction with cellular debris containing fibrin and mucus is also a prominent feature in the pathology of fatal untreated human RSV infection. [29] A recent study showed that poly-IC, a model of viral infection, induced a pro-coagulant state on the endothelium with increased levels of D-dimer, reflecting fibrin turnover. [30] Importantly, our previous collaborative work has also shown significantly *increased* fibrin formation in the airways of asthmatic subjects with proven viral infection. [31]

We also recently reported a chance observation of fibrin formation in the airways of a patient with moderate asthma 5 days before a severe exacerbation requiring hospital admission. Levels of plasma exudation, D-dimers, fibrinogen, plasminogen, and PAI-1 were all orders of magnitude (22–70 fold) higher than those seen in stable moderate or severe asthma. This indicated activation of the extrinsic coagulation cascade in the airways prior to an exacerbation when the patient was asymptomatic and conventional measures of airway dysfunction were normal. [32] We proposed that coagulation factors had in part mediated the exacerbation, or may be biomarkers of early changes in the airways as a prelude to an exacerbation. We have also conducted preliminary analyses of coagulation factors in urine of patients and controls; 6/6 healthy controls were negative, and in asthma, 1/6 stable positive and 5/8 exacerbation samples were strongly positive. We will now improve the lower limit of detection of the assay.

AIMS

(1) To assess whether markers of fibrin turnover are elevated in the sputum, blood and urine during exacerbations of asthma requiring hospital treatment or admission compared to stable disease, and if elevated, whether these factors return to normal after exacerbation.
(2) To assess whether these factors become elevated before an exacerbation in moderate and severe asthma, compare with changes in symptom or lung function prior to exacerbation, and assess (if elevated) the time course of these factors to return to normal after an exacerbation.

The long-term goal is to identify novel targets for asthma therapy and a method for predicting exacerbation of asthma, allowing possible pre-emptive intervention.

METHODS

The proposed study consists of a 2 part study looking at the inflammatory and microbiological changes that occur during exacerbations of asthma and subsequent recovery from exacerbation. In particular we will focus on the changes occurring to the coagulation and fibrinolytic pathways. The study participants will be recruited from the 2 centres, Queen Alexandra Hospital (QAH) in Portsmouth and the North Hampshire Hospital (NHH) in Basingstoke. Further study sites may be enrolled from the NIHR/CSP portfolio website for both parts of the study. Recruitment will also occur from selected local GP surgeries in the Portsmouth area. Participant Information Sheets and study team contact details will be given to subjects by practice staff at the surgery. If potential study subjects are interested in taking part they will be asked to contact the study team directly. The study team will not have direct access to GP asthma clinic registers.

Recruitment for both parts of the study will proceed in parallel. The proposed study period is for 2 years. Ethics approval will be sought from the National Research Ethics Committee. Written informed consent will be obtained from all study participants prior to study entry.

Part 1 – Observational Study

This study will evaluate coagulation factors in blood and urine of 100 subjects during admission to hospital or treatment in the emergency department for an asthma exacerbation. Adult patients (18-70 yrs) will be identified from computerized admission records, and those willing to participate will undergo baseline assessment of lung function and exhaled nitric oxide. Those with a smoking history >20 pack years or a history of COPD, other respiratory disorder or bleeding diatheses will be excluded. We will collect blood, urine and sputum specimens at the time of admission for analysis. Sputum will be collected in a sub-group of 20 patients at admission and when stable (freely expectorated but not induced when exacerbating, but induced when stable). Baseline spirometry and exhaled nitric oxide will be measured and recorded, and peak expiratory flow rate (PEFR) measurements will be recorded twice daily. Any treatment required for the exacerbation will be given in line with British Thoracic Society (BTS) guidelines for the management of asthma. This is an observational study only and treatment will not be influenced by study participation. On discharge from hospital, patients will be asked to continue to record a PEFR diary and review will be arranged at 4 weeks when recovery will be confirmed by history, clinical examination, review of PEFR diary and spirometry. If patients have a smoking history between 10 and 20 pack years, we will also perform full lung function testing to exclude evidence of COPD. At this time repeat blood, urine and sputum samples will be collected for comparative analysis. We will also perform skin prick tests for common aeroallergens and repeat exhaled nitric oxide testing. Those remaining unwell (i.e. PEFR not returned to >80% predicted best) will provide samples but return after another 4 weeks for further sample collection until the exacerbation has resolved.

Samples will be analysed for differential inflammatory cell counts, markers of fibrinogenesis and fibrinolysis, protein markers of inflammation and virology.

Part 2 – Longitudinal Study

Forty asthmatic subjects (20 moderate, 20 severe) confirmed by objective assessments (e.g. variable airflow obstruction and/or AHR) will be identified from the Asthma Registry at QAH and the general respiratory clinics at NHH, alongside participants identified by local GP surgeries. Individuals participating in part 1 of the study will be eligible to apply. The 1st baseline assessment will include a clinical assessment, baseline spirometry, blood, urine, skin prick tests for common aeroallergens, exhaled nitric oxide and sputum sampling. They will also be asked to monitor and record their PEFR twice daily and provide three times weekly early morning urine samples until a significant exacerbation is reported. We will arrange for a weekly collection or drop off point for urine samples, tailored to the individual participant.

When an individual experiences symptoms consistent with an exacerbation of their asthma or a significant drop in their PEFR, >20% from their best recorded, and requiring a course of oral corticosteroids, they will be asked to attend the outpatient department for a 2nd assessment. Treatment for an exacerbation will be given by the investigators or the General Practitioner (GP) in accordance with BTS guidelines and usual practice for the individual asthmatic as per their individual asthma management plan. At this appointment further specimens will be taken of blood and sputum, and spirometry and exhaled nitric oxide will be measured. A combination of symptoms, PEFR and spirometry will be used to confirm an asthma exacerbation. Daily diary and thrice-weekly urine sampling will continue for 4 weeks until the final blood and urine sampling visit. This 3rd and final visit will be scheduled 4 weeks after the exacerbation for assessment, examination of PEFR diary, repeat spirometry, repeat exhaled nitric oxide, blood sampling, and sputum sampling, to confirm recovery from exacerbation. Throughout this period three times weekly urine sampling and PEFR diary recordings will be taken. Those remaining unwell will provide samples but will return after a further 4 weeks for repeat assessment.

Each subject will be kept under follow up until they exacerbate and subsequent recovery is confirmed. Initial samples will be collected and tested on a weekly basis as they are required to be processed while fresh. Further analysis of those samples will be based on the pattern of responses and signal duration. Samples will be tested for the markers listed in part 1 and sputum will again only be collected if an individual is able to spontaneously expectorate during exacerbation but induced at baseline and recovery. All sputum induction will be performed as in our previous work and in accordance with hospital protocol. They will thus be prospectively assessed for changes in coagulation factors in blood and urine before, during and after exacerbation. Disease severity will be categorized by level of inhaled and oral corticosteroid requirement (see below - GINA criteria and in keeping with our previous work including exclusion criteria).

Inclusion Criteria

1. A diagnosis of asthma >1 year
2. Chronic moderate persistent disease [33] requiring medium daily doses of inhaled glucocorticosteroids (>500 – 1000 micrograms of beclomethasone dipropionate (BDP) or equivalent) for moderate asthma group.
3. Severe persistent disease [33] requiring high daily doses of inhaled glucocorticosteroids (>1000 – 2000 micrograms of BDP or equivalent) and/or regular oral corticosteroid use.
4. For part 1, an acute exacerbation of asthma requiring admission to hospital for treatment of that exacerbation.
5. Age 18-70 years.

Exclusion criteria

1. Current or ex-smokers with >20 pack year history
2. Significant cardiopulmonary co-morbidities
3. Clinically significant neurological, renal, endocrine, gastrointestinal, hepatic or haematological abnormalities uncontrolled with standard treatment.
4. Regular use of an anticoagulant
5. Bleeding diathesis
6. Alcohol and recreational drug abuse
7. Treatment with other immunomodulators
8. Concurrent participation in another study
9. Abnormal chest xray
10. For part 2: an asthma exacerbation in the 4 weeks prior to study recruitment (visit 1)

Analyses

Blood, sputum (where applicable) and urine will be tested for differential cell counts, coagulation factors, chemokines, CRP, viral PCR screen and markers of fibrin synthesis and degradation using commercially available immunoassays (detailed protocols available upon request – see our earlier studies).

Statistics

Based on our preliminary studies, we observed 2 to 72-fold increases in pre-exacerbation levels of coagulation factors in sputum compared to stable disease, and we expect to find similar differences in blood and urine. We performed simulations after logarithmic transformation at 90% power (5% significance) to detect between 1-5 fold differences in coagulation factors in a 1:1 ratio. We anticipate 100 subjects will provide sufficient power to detect these differences. Anticipating potential subgroup analyses, we plan to perform an interim analysis after recruiting 50 subjects, and may increase our target beyond 100 patients to allow successful subgroup comparison. This interim analysis will also provide important preliminary data to guide any necessary sample size increase for part 2.

The longitudinal study is pilot work and the numbers based on experience of intensive prospective follow up of adults with asthma over about 4 months per subject. Standard tests of significance (parametric and non-parametric) will be used to compare between groups for Aim (1). ANOVA will be used (Aim (2)) to examine exacerbations preceded by a rise in coagulation factors compared to those that are not. A minimum of 12 patients in each sub-group (moderate and severe) are required for useful ANOVA analysis, we plan to recruit 40 subjects (20 moderate and 20 severe). This should allow for subject withdrawal, failure to exacerbate and failure to record differences between exacerbation and stable disease.

Timeline

The project will be completed in 1 year including sample collection and laboratory analyses (>700 admissions per year and asthma registry >300 patients at QA Hospital, Portsmouth).

Adverse Event Reporting

Adverse events will be reported as directed by the local NHS Trust operating procedures at both study sites. Patients will be recruited following the initial index hospital admission (which will thus not be reported as an SAE). Patients admitted to hospital with an exacerbation will by definition have uncontrolled asthma, which is recognised clinically and epidemiologically as high risk for further admissions for acute asthma. Any such further admissions will be reported as serious adverse events, even though patients are not given

any investigational medicinal product, as this is simply an observational study involving the collection of blood, sputum and urine.

Costs and Future Plans

This project has attracted funding from the University of Portsmouth Innovations Fund, the salary of a Clinical Research fellow is secured for 2 years, and Oxoid Ltd. will provide the urinary assays. Asthma UK has awarded this study a charitable research grant. If we confirm differences of fibrin turnover in blood or urine, we may develop urinary biomarkers that reflect disease control. We will then apply for NIHR funding to evaluate the predictive value of the assay in larger groups of subjects.

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12 August 2010

Dr Anoop J Chauhan
Consultant Respiratory Physician
Portsmouth NHS Trust
The Respiratory Centre, Trafalgar Building
Queen Alexandra Hospital, Cosham
Portsmouth
PO6 3LY

Dear Dr Chauhan

Study Title: **Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?**

REC reference number: **10/H0505/59**

Thank you for your letter of 29 July 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. I will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to

the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where the only involvement of the NHS organisation is as a Participant Identification Centre (PIC), management permission for research is not required but the R&D office should be notified of the study and agree to the organisation's involvement. Guidance on procedures for PICs is available in IRAS. Further advice should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Investigator CV	A Chauhan	28 August 2009
Investigator CV	J Owen	11 May 2010
Investigator CV	J Shute	30 April 2010
Protocol	1.2	26 July 2010
Protocol Flowchart Part 1	1.1	11 May 2010
Peak flow and symptom record	1.1	11 May 2010
Evidence of peer review: K McKinlay		14 May 2010
Induced Sputum Test Information Sheet	1.2	28 July 2010
Induced Sputum Protocol	1.2	28 July 2010
REC application		17 May 2010
Covering Letter		11 May 2010
Advertisement	1.1	11 May 2010
Letter of invitation to participant	Part 2, v1.2	28 July 2010
Letter of invitation to participant	Part 2 BNHFT, v1.2	28 July 2010
GP/Consultant Information Sheets	1.2	28 July 2010
GP/Consultant Information Sheets	BNHFT, v1.2	28 July 2010
Participant Consent Form: BNHFT	1.2	28 July 2010
Response to Request for Further Information		29 July 2010
Participant Information Sheet: Part 1 - Observational	1.2	28 July 2010
Participant Information Sheet: Part 2 - Longitudinal	BNHFT, v1.2	28 July 2010
Participant Information Sheet: Part 1 - Observational	BNHFT, v1.2	28 July 2010
Protocol: Flowchart Part 2	1.1	11 May 2010
Evidence of peer review: Asthma UK		12 May 2010
Participant Information Sheet: Part 2 - Longitudinal	1.2	28 July 2010
Participant Consent Form: Induced Sputum	1.2	28 July 2010
Participant Consent Form: Induced Sputum	BNHFT, v1.2	28 July 2010

Participant Consent Form	1.2	28 July 2010
Case Record Form - Initial Visit	1.2	28 July 2010
Letter from Statistician		27 July 2010

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk

10/H0505/59	**Please quote this number on all correspondence**

Yours sincerely

pp

Professor Nigel Weilman
Chair

Email: scsha.berksrec@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: Kate Greenwood, The Research and Development department, Portsmouth Hospitals NHS Trust

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

Appendix 5 - PHT R&D (study sponsor) approval letter 27/09/2011

Portsmouth Hospitals NHS
NHS Trust

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Fax: 023 9228 6037
Web: www.porthosp.nhs.uk

Dr Jonathan Owen
Respiratory Department
Queen Alexandra Hospital
Cosham
Portsmouth
PO6 3LY

27 September 2011

Dear Dr Owen,

Re: **Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?**

PI: **Dr Jonathan Owen** Research Office No: **PHT/2011/02ST**

As requested I write to confirm receipt of your email and documents, dated 20 September 2011 concerning an amendment to the above-mentioned trial.

As Sponsor, Portsmouth Hospitals NHS Trust confirm that we are happy for you to contact patients, as identified by the Portsdown Group Practice, for the purpose of recruitment to your study.

The following documents associated with the amendment have been reviewed for local impacts:

Document	Version	Dated
Surgery Invite Letter	1.3	14 September 2010
Letter of Approval - Recruitment from PIC	HIOW Shared RM&G Service	19 August 2011
Notice of Amendment	Amendment 01	28 October 2010
Ethics Approval Letter	Amendment 01 (inc. Surgery Invite Letter)	30 November 2010

I confirm continued NHS organisation permission based on the amendment details above.

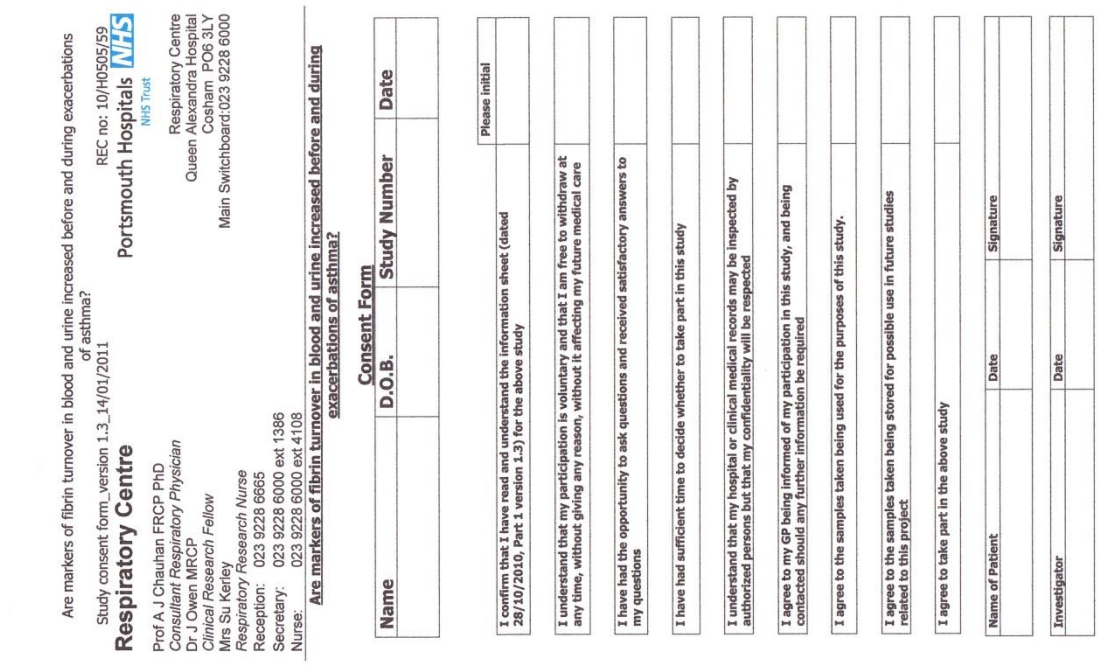
Yours sincerely

Kate Greenwood

Kate Greenwood
Research Manager

CC. CI: Prof Anoop Chauhan (Anoop.chauhan@porthosp.nhs.uk)

Appendix 6 – Consent form Part 1



Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

Study consent form_version 1.3_14/01/2011

REC no: 10/H0505/59

Respiratory Centre

Portsmouth Hospitals NHS Trust

Prof A J Chauhan FRCP PhD — *Consultant Respiratory Physician* — Respiratory Centre
Dr J Owen MRCP — *Clinical Research Fellow* — Queen Alexandra Hospital
Mrs Su Kerley — *Respiratory Research Nurse* — Cosham PO6 3LY
Reception: 023 9228 6665
Secretary: 023 9228 6000 ext 1386 — Main Switchboard:023 9228 6000
Nurse: 023 9228 6000 ext 4108

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

Consent Form

Name	D.O.B.	Study Number	Date

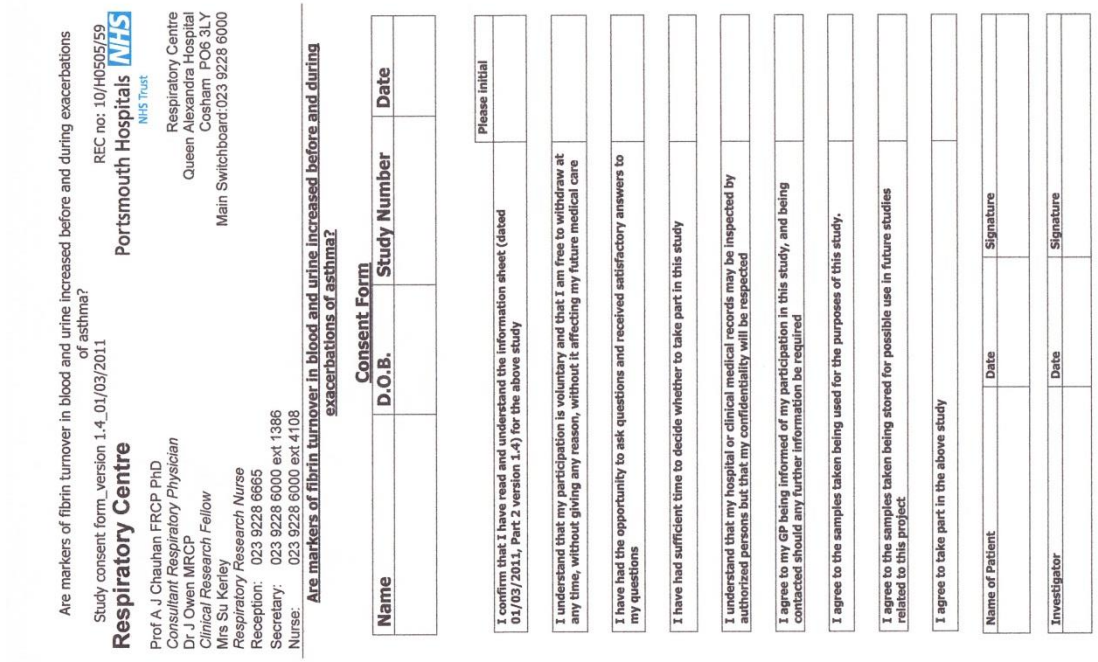
	Please initial
I confirm that I have read and understand the information sheet (dated 28/10/2010, Part 1 version 1.3) for the above study	
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without it affecting my future medical care	
I have had the opportunity to ask questions and received satisfactory answers to my questions	
I have had sufficient time to decide whether to take part in this study	
I understand that my hospital or clinical medical records may be inspected by authorized persons but that my confidentiality will be respected	
I agree to my GP being informed of my participation in this study, and being contacted should any further information be required	
I agree to the samples taken being used for the purposes of this study.	
I agree to the samples taken being stored for possible use in future studies related to this project	
I agree to take part in the above study	

Name of Patient	Date	Signature

Investigator	Date	Signature

1 copy for patient, 1 copy for researcher, 1 copy for medical records.

Appendix 7 – Consent form Part 2



Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

Study consent form_version 1.4_01/03/2011

REC no: 10/H0505/59

Respiratory Centre

Portsmouth Hospitals NHS Trust

Prof A J Chauhan FRCP PhD — *Consultant Respiratory Physician* — Respiratory Centre
Dr J Owen MRCP — *Clinical Research Fellow* — Queen Alexandra Hospital
Mrs Su Kerley — *Respiratory Research Nurse* — Cosham PO6 3LY
Reception: 023 9228 6665
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Nurse: 023 9228 6000 ext 4108

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

Consent Form

Name	D.O.B.	Study Number	Date

	Please initial
I confirm that I have read and understand the information sheet (dated 01/03/2011, Part 2 version 1.4) for the above study	
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without it affecting my future medical care	
I have had the opportunity to ask questions and received satisfactory answers to my questions	
I have had sufficient time to decide whether to take part in this study	
I understand that my hospital or clinical medical records may be inspected by authorized persons but that my confidentiality will be respected	
I agree to my GP being informed of my participation in this study, and being contacted should any further information be required	
I agree to the samples taken being used for the purposes of this study.	
I agree to the samples taken being stored for possible use in future studies related to this project	
I agree to take part in the above study	

Name of Patient	Date	Signature

Investigator	Date	Signature

1 copy for patient, 1 copy for researcher, 1 copy for medical records.

Appendix 8 – MAU/ED study poster

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma? – Part 1 – Observational study

ED/MAU poster_version 1.1_11/5/2010 REC no: 10/H0505/59

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Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?
Part 1 – Observational Study

Is your patient having an **asthma exacerbation?**

They may be eligible for our study

Please bleep Dr Jonathan Owen on 0164 or contact Su Kerley Research Nurse on extension 4108

Thank you!

Respiratory Centre

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NHS Trust

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Respiratory Research Nurse

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Reception: 023 9228 6665 Email: Sumita.Kerley2@porthosp.nhs.uk
Secretary: 023 9228 6000 ext 1386 Email: Jonathan.Owen2@porthosp.nhs.uk
Nurse: 023 9228 6000 ext 4108

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

Part 2 – Longitudinal Study

Date

Address

Dear

I am writing to invite you to take part in a research study. We are looking at chemical changes that occur in the blood and urine of people with asthma when they have an attack. In particular we are interested in changes in markers of inflammation in urine, which may change just before an asthma attack and possibly even before symptoms of an attack begin.

We are looking at people with moderate to severe asthma and you fall into one of these categories and may be eligible to take part in this research.

For this study we will arrange to see you for an initial assessment, then ask you to keep a peak flow diary and 3 times weekly urine samples (which we will collect) until you have an asthma attack, when we will arrange to see you again. You will then be asked to collect urine and keep your diary for 4 more weeks until we see you for a 3rd and final appointment to confirm you have recovered.

If you are interested in participating then please contact myself or Su Kerley at the Respiratory Centre on the numbers/email addresses above and we will provide you with more information and arrange an appointment to see us if you wish.

Thank you for taking the time to read this letter, we look forward to hearing from you.

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?
Invitation letter_part 2_version 1.2_28/07/2010 REC no: 10/H0505/59

Yours sincerely

Dr Jonathan Owen

Respiratory Centre

Portsmouth Hospitals NHS
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Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

Dear Colleagues

We are currently undertaking a research study looking at the coagulation cascade as a marker of inflammation in asthma. The study is being run by The Respiratory Centre at QAH in collaboration with the University of Portsmouth and will be submitted, upon completion, by Dr Owen towards an MD thesis from the university.

This is an exciting area of research and is a continuation of work previously carried out locally by our group that has been published in international journals such as Thorax, Asthma and Clinical Experimental Allergy. This latest project proposal has been awarded a charitable grant from Asthma UK following peer review by expert, lay and patient members.

Background

When epithelial damage occurs in the airways there is activation of the extrinsic coagulation cascade via tissue factor and plasma exudation into the airways that helps to repair the damaged epithelium. In the normal airway fibrinolysis is favoured over fibrinogenesis and once epithelial damage has been repaired the fibrin plug is removed. In asthmatic airways this fibrinolytic and fibrinogenic balance is disturbed.

What our study is looking at

In this latest study we plan to look at asthmatics during exacerbations. We plan to recruit patients prospectively and follow them up until they exacerbate and then through to recovery. We will compare blood, sputum, urine, spirometry and exhaled nitric oxide at an initial assessment, when they exacerbate and again after recovery. Between assessments we will collect thrice weekly urine samples, and peak flow data and symptom data. We

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?
Surgery invite_version 1.3_14/09/2010 REC no: 10/H0505/59

hope to see changes in urinary coagulation factors which correspond with clinical evidence of exacerbation that may lead to future biomarkers, potential treatment targets and possibly predictors of imminent exacerbation.

This is an observational study only; we will not be testing any treatments on your patients.

Any treatment provided by our team will be in line with British Thoracic Society (BTS) guidelines or in line with individual treatment plans a patient may already have.

Who do we want?

We plan to recruit 20 severe and 20 moderate asthmatics. Although we see many severe asthmatics at the Respiratory Centre, moderate asthmatics are generally managed in the community and this is where we particularly need your help.

We are looking for people with moderate asthma who are on a dose of inhaled corticosteroids between 500 and 1000 mcg beclomethasone dipropionate (BDP) or equivalent. For example, Clenil 250 MDI 2 puffs BD and Symbicort 200/6 2 puffs BD fall within this bracket. Of note, QVAR and Fluticasone are essentially twice the strength of BDP so a QVAR 100 MDI 2 puffs BD would also fall into this bracket.

If you also see more severe asthmatics on larger doses of corticosteroids who might be interested in taking part we will happily see them as well.

What are we asking you to do?

We would be extremely grateful if you would consider our study when seeing asthmatic patients and if you think they may be interested give them a Participant Information Sheet (PIS) which I enclose and ask them to call our department if they are keen to take part.

We have a list of exclusion criteria which is on the enclosed study protocol. If there is doubt we would rather assess individuals who are willing to take part and review whether they are suitable at this assessment.

What is in it for your patients?

We are offering the opportunity to take part in a large research project which will help to improve our understanding of asthma, may reveal a method for predicting and monitoring asthma exacerbations, and may identify potential targets for future therapies. Although we cannot offer financial rewards for participation, we will reimburse travel expenses to ensure patients are not out-of-pocket. Patients will also undergo a specialist respiratory assessment

including spirometry, exhaled nitric oxide testing, allergy skin prick testing, blood and urine tests as outlined above. They will also undergo sputum induction during the initial visit and recovery. The results of these tests will be made available upon study completion if requested and may guide your patient's future care.

Summary

- We are looking for asthmatics who may be interested in taking part in asthma research
- This is not a drug trial, it is an observational study only
- We are looking for people who need more than 500mcg of inhaled corticosteroids a day
- This study is being supported by a charitable grant from Asthma UK

If you have any questions please do not hesitate to get in touch, I enclose a copy of the full study protocol and the PIS.

We are most grateful for your help.

Yours faithfully

Dr Jonathan Owen
Clinical Research Fellow
Bleep 0164
07773 353413
Jonathan.Owen2@porthosp.nhs.uk

Past work from our group for your reference:

Brims FJ, Chauhan AJ, Higgins B, Shute JK. Upregulation of the extrinsic coagulation pathway in acute asthma – a case study. J Asthma 2010 Aug;47(6):695-8.

Brims FJ, Chauhan AJ, Higgins B, Shute JK. Coagulation factors in the airways of moderate and severe asthma and the effect of inhaled steroids. Thorax 2009;64:1037-1043

Perrio MJ, Ewen D, Trevethick MA, Salmon GP, Shute JK. Fibrin formation by wounded bronchial epithelial cell layers in vitro is essential for normal epithelial repair and independent of plasma proteins. Clin Exp Allergy 2007;37:1688-700.

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Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

Part 1 – Observational study

Participant Information Sheet

Introduction

We invite you to take part in a research study looking at the inflammation that occurs in people with asthma. Before you agree to take part it is important that you understand the role you will play. This letter outlines the project plan and hopefully answers any questions you may have. This project is being completed as part of a postgraduate degree by Dr Owen. We hope to recruit at least 100 participants to this study. Please discuss it with others if you wish or feel free to ask the investigators any further questions you might have.

Study outline

In people with asthma we know that the disease is driven by inflammation in the airways. Past research has found changes that occur in blood, urine and sputum of people with asthma compared with people who don't have asthma. These studies looked mainly at people who were well with their asthma. We think that if you have an asthma attack there will be more dramatic changes seen in those blood, urine and sputum tests. In particular we suspect that these changes begin before you feel unwell and may be a way of predicting an asthma attack in the future.

We want to take samples of urine, blood and sputum from people who suffer an attack of their asthma and look at the inflammatory changes that occur in these samples. We then want to repeat those tests after recovery from the attack to look at the differences that may occur.

We will not be testing any new drugs or treatments on you.

Why have I been chosen to take part?

You have unfortunately had an attack of your asthma which was bad enough for you to need hospital treatment. The inflammatory changes we are looking for may already be taking place and we should be able to look at these changes if we test your blood, urine, and if coughing, your sputum. As you recover from your asthma we can look again at repeat samples to easily compare the differences that occur between an asthma attack and stable controlled asthma.

Exclusion Criteria

In order to minimise any possible inaccuracies with the study and prevent any problems with the tests we are performing, we are not including people with the following:

1. A history of smoking 20 cigarettes a day for 20 years or more (or an equivalent amount).
2. Any significant heart or lung disease other than asthma.
3. Significant nerve, kidney, hormone, bowel, liver or blood disease that is not controlled with treatment.
4. People taking warfarin or similar blood-thinning drugs.
5. People with an abnormal bleeding tendency such as haemophilia.
6. Excessive alcohol use or recreational drug use.
7. Treatment with drugs that alter the immune system.
8. Current involvement in another clinical trial.
9. An abnormal chest xray.

If you think you may fall into one of these categories but are interested in taking part, please discuss it with the investigators.

Do I have to take part?

It is up to you to decide. We will provide you with the details of the study and this information leaflet. If you decide to be included we will ask you to sign a consent form to say you agree to take part. If you choose not to take part your usual care will not change in any way. If you agree to be involved but change your mind at a later date you may withdraw at any time without giving a reason. Again, this will not change the standard of care you receive in the future. Any information obtained before you leave the study may still be used in the final data analysis.

What will the study involve?

In short, the study will involve an extra blood test and urine test while you are in hospital that would not normally be taken, and possibly a sputum test.

We will ask you to monitor and record your peak flow for 4 weeks then be seen at the hospital for an examination and repeat tests.

During your hospital stay, alongside the usual medical care you will receive, you will be seen by a research doctor. They will take an extra blood and urine test and if you are coughing up any sputum, a sample may be taken of this too. You will be given a peak flow meter and asked to record your peak flow twice a day in a diary provided. You will also have breathing tests during your stay.

On discharge we would like you to continue to monitor and record your peak flow. We will then arrange to see you at the hospital when you are better, 4 weeks after going home. At this visit you will see a doctor who will take a medical history and perform a medical examination. We will also repeat a urine test, blood test and possibly a sputum test. If you can't cough up any sputum, we may ask you to inhale some salt water to try and make you cough some sputum for a sample. This will be explained in more detail on the day and we will go through a separate consent form for this test. Your breathing test will be repeated and we will collect your peak flow diary results. We will also perform a skin prick test to look for common allergies and an extra blowing test looking for evidence of inflammation in your lungs by measuring the amount of nitric oxide as you breathe out. If you have not recovered after 4 weeks, we may ask you to continue your peak flow diary and to come back again 4 weeks later for another check up and tests.

What are the benefits of taking part?

On top of the usual care you would receive at the hospital, you will have an extra appointment on going home where you will have a thorough review of your asthma. You will be taking part in research that will help our understanding of asthma. The study will help to guide future asthma management and possibly point towards new ways of treating the disease. We will reimburse any expenses you may incur in travelling to the hospital for the purposes of the study.

Are there any risks from taking part?

Most of this study involves taking samples that would usually be tested during a hospital visit and are of no risk to you. The only test with a small risk involved is when inducing sputum, which can irritate your airways and rarely can cause an asthma attack. This will be explained in detail and you will be asked to sign a separate consent for this. This test is performed in a controlled way under the supervision of doctors who can treat any irritation that may occur.

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?
Participant Information Sheet_part1_version 1.3_28/10/2010 REC no: 10/H0505/59

Involvement of your General Practitioner (GP)

With your permission, we will write to your GP to inform them you are taking part in this study. We will send them information outlining the study and tell them any information we find that they may need to help you with your care. When your part in the study is complete we will let them know.

Will the information I provide be confidential?

Yes. All information collected will be made anonymous before it leaves the confines of the hospital or GP surgery in accordance with usual NHS practice.

What will happen to the samples I provide?

Any blood, urine and sputum samples you provide will be tested to look for markers of inflammation in relation to your asthma. Once we have completed testing for this study the samples may be stored and used for future studies. Future studies using your samples would be a continuation of the work we are currently doing looking at asthma and inflammation. Any new studies testing those samples will have been approved by an ethics committee to ensure any testing is appropriate. All samples kept will be stored anonymously. The consent form you sign when agreeing to take part in the study will include a section agreeing for your samples to be stored in this way and you can choose to opt out of this sample storage if you wish.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The researchers will do their best to answer any questions you may have. If you remain unhappy and wish to make a formal complaint you can do this through the NHS complaints procedure, details of which can be obtained from the hospital. In the unlikely event that something does go wrong and you are harmed due to someone's negligence then you may have grounds for legal action for compensation but you may have to pay your legal costs.

Who has given approval for this study to proceed?

This study was given a favourable ethical opinion for conduct by the Berkshire Research Ethics Committee.

What happens once the study has ended?

The information obtained will be analysed, presented to health professionals at conferences and may be published in scientific journals. Your individual details will not be identified in any report or publication. We will inform you

of the results of the study by letter unless you specifically request that we do not send you this information.

Who is organising and funding the research?

The study is being organised by the Respiratory Departments at Portsmouth and Basingstoke, and the University of Portsmouth. A research grant has been awarded for this study from the charity Asthma UK. This study is not being funded by the pharmaceutical industry.

Further information

If you would like any further information not provided in this letter then please do not hesitate to contact the investigating team and we will be happy to answer you queries. Please contact either Mrs Sue Kerley, Research Nurse, or Dr Jonathan Owen, Clinical Research Fellow via the Respiratory Centre at QAH. For advice regarding this research from a respiratory specialist not directly involved with the study please contact Mrs Chris Fehrenbach, Respiratory Matron at the Respiratory Centre. Further general information regarding the process of research can be obtained from the Medical Research Council (MRC) website at www.mrc.ac.uk.

Thank you for taking the time to read this information letter.

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Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

Part 2 – Longitudinal study

Participant Information Sheet

Introduction

We invite you to take part in a research study looking at the inflammation that occurs in people with asthma. Before you agree to take part it is important that you understand the role you will play. This letter outlines the project plan and hopefully answers any questions you may have. This project is being completed as part of a postgraduate degree by Dr Owen. We hope to recruit 40 participants to this study. Please discuss it with others if you wish or feel free to ask the investigators any further questions you might have.

Study outline

In people with asthma we know that the disease is driven by inflammation in the airways. Past research has found changes that occur in blood, urine and sputum of people with asthma compared with people who don't have asthma. These studies looked mainly at people who were well with their asthma. We think that if you have an asthma attack there will be more dramatic changes seen in those blood, urine and sputum tests. In particular we suspect that these changes begin before you feel unwell and may be a way of predicting an asthma attack in the future.

We want to monitor people with asthma taking their usual medicine and living their normal life. If they have an asthma attack we want them to be treated as they normally would be to get better as soon as possible, and see them to make sure they have recovered.

During this time we want to monitor people's peak flows and collect urine samples three times a week. When they become unwell and again when they are better we plan to take blood and collect sputum.

We will not be testing any new drugs or treatments on you.

Why have I been chosen to take part?

You have been invited to take part as you need to take regular steroid medication for your asthma. If you need a higher dose of inhaled steroid, or regular steroid tablets you fall into the moderate to severe category of asthma which is who we are interested in looking at.

Exclusion Criteria

In order to minimise any possible inaccuracies with the study and prevent any problems with the tests we are performing, we are not including people with the following:

1. A history of smoking 20 cigarettes a day for 20 years or more (or an equivalent amount).
2. Any significant heart or lung disease other than asthma.
3. Significant nerve, kidney, hormone, bowel, liver or blood disease that is not controlled with treatment.
4. People taking warfarin or similar blood-thinning drugs.
5. People with an abnormal bleeding tendency such as haemophilia.
6. Excessive alcohol use or recreational drug use.
7. Treatment with drugs that alter the immune system.
8. Current involvement in another clinical trial.
9. An abnormal chest xray.

In addition, we will need to wait at least 4 weeks after any recent attack that needed steroids or an admission to hospital before recruiting you to the study.

If you think you may fall into one of these categories but are interested in taking part, please discuss it with the investigators.

Do I have to take part?

It is up to you to decide. We will provide you with the details of the study and this information leaflet. If you decide to be included we will ask you to sign a consent form to say you agree to take part. If you choose not to take part your usual care will not change in any way. If you agree to be involved but change your mind at a later date you may withdraw at any time without giving a reason. Again, this will not change the standard of care you receive

in the future. Any information obtained before you leave the study may still be used in the final data analysis.

What will the study involve?

In short, the study will involve 3 visits to hospital, monitoring your Peak Flow at home and providing regular urine specimens.

The first visit to hospital will be with a doctor at the Asthma Clinic at Queen Alexandra Hospital (QAH) in the Respiratory department. At this appointment you will see a doctor and have a thorough health check including taking a medical history, a physical examination, breathing tests, a blood test, urine test and skin prick tests for common allergies. The breathing tests will include an exhaled nitric oxide test which involves blowing into a machine which looks at inflammation in your lungs. We will also ask you to give us a sputum sample. If you can't cough any up, we may ask you to inhale some salt water to try and make you cough some sputum for a sample. This will be explained in more detail on the day and we will go through a separate consent form for this test. We will send you home with a peak flow meter, a peak flow diary, some urine pots and a container or bags to keep the urine pots in.

At home we will ask you to measure and record your peak flow twice a day, and provide a urine sample first thing in the morning three times a week. We will provide you with clean containers or bags for the urine sample pots and ask you to store the samples in a fridge. We will arrange a weekly drop off or collection for these samples that is convenient to yourself and our team.

During the study period should you become unwell with an asthma attack you will be asked to contact the Respiratory Centre at QAH. We will arrange to see you and repeat an examination, breathing tests, a blood test and a sputum test if coughing. If you need treatment for your attack you will take this as you normally would.

We will arrange to see you for a final visit 4 weeks after you became unwell to see that you have recovered. During those 4 weeks we will ask you to continue to provide urine specimens and monitor your peak flow. At this final appointment we will again repeat the examinations and tests, including induced sputum, and collect your peak flow diary and final urine samples. This will be the end of your part in the study.

What are the benefits of taking part?

You will have a thorough assessment of your asthma. You will be taking part in research that will help our understanding of asthma. The study will help to guide future asthma management and possibly point towards new ways of

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?
Participant Information Sheet_part 2_version 1.4_01/03/2011 REC no: 10/H0505/59

treating the disease. We will reimburse any expenses you may incur in travelling to the hospital for the purposes of the study.

Are there any risks from taking part?

Most of this study involves taking samples that would usually be tested during a hospital visit and are of no risk to you. The only test with a small risk involved is when inducing sputum, which can irritate your airways and rarely can cause an asthma attack. This will be explained in detail and you will be asked to sign a separate consent for this. This test is performed in a controlled way under the supervision of doctors who can treat any irritation that may occur.

Involvement of your General Practitioner (GP)

With your permission, we will write to your GP to inform them you are taking part in this study. We will send them information outlining the study and will communicate with them any information we find that they may need to help you with your care. When your part in the study is complete we will let them know.

Will the information I provide be confidential?

Yes. All information collected will be made anonymous before it leaves the confines of the hospital or GP surgery in accordance with usual NHS practice.

What will happen to the samples I provide?

Any blood, urine and sputum samples you provide will be tested to look for markers of inflammation in relation to your asthma. Once we have completed testing for this study the samples may be stored and used for future studies. Future studies using your samples would be a continuation of the work we are currently doing looking at asthma and inflammation. Any new studies testing those samples will have been approved by an ethics committee to ensure any testing is appropriate. All samples kept will be stored anonymously. The consent form you sign when agreeing to take part in the study will include a section agreeing for your samples to be stored in this way and you can choose to opt out of this sample storage if you wish.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The researchers will do their best to answer any questions you may have. If you remain unhappy and wish to make a formal complaint you can do this through the NHS complaints procedure, details of which can be obtained from the hospital. In the unlikely event that something does go wrong and you are harmed due to

someone's negligence then you may have grounds for legal action for compensation but you may have to pay your legal costs.

Who has given approval for this study to proceed?

This study was given a favourable ethical opinion for conduct by the Berkshire Research Ethics Committee.

What happens once the study has ended?

The information obtained will be analysed, presented to health professionals at conferences and may be published in scientific journals. Your individual details will not be identified in any report or publication. We will inform you of the results of the study by letter unless you specifically request that we do not send you this information.

Who is organising and funding the research?

The study is being organised by the Respiratory Departments at Portsmouth and Basingstoke, and the University of Portsmouth. A research grant has been awarded for this study from the charity Asthma UK. This study is not being funded by the pharmaceutical industry.

Further information

If you would like any further information not provided in this letter then please do not hesitate to contact the investigating team and we will be happy to answer you queries. Please contact either Mrs Sue Kerley, Research Nurse, or Dr Jonathan Owen, Clinical Research Fellow via the Respiratory Centre at QAH. For advice regarding this research from a respiratory specialist not directly involved with the study please contact Mrs Chris Fehrenbach, Respiratory Matron at the Respiratory Centre. Further general information regarding the process of research can be obtained from the Medical Research Council (MRC) website at www.mrc.ac.uk.

Thank you for taking the time to read this information letter.

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Addressograph label

Name

Hospital No.

D.O.B.

Address

Date:_____

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

GP Information Letter

Dear Doctor

Your patient above has agreed to take part in this study looking at the inflammatory changes that occur in asthma.

This letter is to outline the role your patient will play in the study. If after reading this you have any further questions, please contact either Sue Kerley Research Nurse, or Dr Jonathan Owen at the Respiratory Centre, Queen Alexandra Hospital.

The study comprises 2 parts and patients may be recruited to either one, or both parts which are described below.

Your patient has agreed to participate in part(s) _____of this study.

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?
GP letter_version 1.2_28/07/2010 REC no: 10/H0505/59

Background

It has been found that there are significant changes which occur in the coagulation and fibrinolysis pathways in the airways in asthma. These changes can be observed by blood, urine and sputum analysis. Previous work has looked at stable asthmatics in the outpatient setting. In a past study by our group, we observed that markers of fibrin turnover increased in sputum several days prior to an acute exacerbation of asthma.

The aims of our current study are to look at these markers in more detail and compare asthma exacerbation to recovery. This study comprises 2 parts. The first will recruit 100 patients who are admitted to hospital for an exacerbation of their asthma. We will sample urine, blood and possibly sputum during this admission along with spirometric and exhaled nitric oxide testing. On discharge they will keep a peak flow (PEFR) diary. Individuals will be reviewed in outpatients 4 weeks after admission to assess recovery and repeat the above tests. This will conclude part one of the study.

Part 2 will recruit 40 patients who have moderate to severe asthma and prospectively follow them up until they exacerbate. The initial assessment will comprise blood, urine, sputum and exhaled nitric oxide testing along with spirometry. Sputum induction with nebulised hypertonic saline may be performed in line with the trust protocol which includes a period of patient observation to ensure significant bronchospasm does not occur. Again they will be asked to record a PEFR diary. Alongside this we will collect early morning urine three times weekly for analysis. When they exacerbate they will be reviewed and the initial assessment will be repeated. Follow up will continue with PEFR and urine testing until the final review 4 weeks after exacerbation when blood, urine, exhaled nitric oxide, induced sputum and spirometric testing will again be performed. This will conclude part 2 of the study.

This is an observational study and we are not trialling any new medications or treatment approaches. Any adjustments to therapy will be in accordance with British Thoracic Society (BTS) guidelines for asthma management. As part of our cohort have severe asthma, their individual management may fall beyond the scope of the BTS guidelines. In these circumstances any treatment changes will be in accordance with the usual practice of the respiratory physicians involved and will be guided by the individual's clinical condition and not influenced by the research protocol.

We will inform you of any changes to treatment that may be required for your patient during an exacerbation (e.g. oral corticosteroid therapy) and would be very happy to discuss any alternative treatment options you suggest for your patient.

It is important to highlight that we will not be testing any new medication or therapy on your patient.

Please find enclosed a copy of the information letter given to your patient which further outlines their involvement.

Yours faithfully

Dr Jonathan Owen
Clinical Research Fellow

Case Record Form - Admission

Addressograph
Name & Hospital No.
Address
D.O.B.

Subject's study number:

Date Seen: ___/___/_____

Tel No:

Next of Kin: N.O.K. Tel No:

Inclusion Criteria:	**Yes**	**No**
• A diagnosis of asthma >1 year	☐	☐
• An acute exacerbation of asthma requiring admission to hospital for treatment of that exacerbation.	☐	☐
• Age 18-70 years	☐	☐

Exclusion Criteria:	**Yes**	**No**
• Current or ex-smokers with >20 pack year history	☐	☐
• Significant cardiopulmonary co-morbidities	☐	☐
• Clinically significant neurological, renal, endocrine, gastrointestinal, hepatic or haematological abnormalities uncontrolled with standard treatment.	☐	☐
• Regular use of an anticoagulant	☐	☐
• Bleeding diatheses	☐	☐
• Alcohol and recreational drug abuse	☐	☐
• Treatment with other immunomodulators	☐	☐
• Concurrent participation in another interventional study	☐	☐
• Abnormal chest xray	☐	☐

Signature:_____ Date:___/___/_____ 1

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma? – Part 1 – Observational Study
Case Record Form_admission_version 1.3_01/03/2011 REC no: 10/H0505/59

Addressograph
Name & Hospital No.
Address
D.O.B.

Subject's study number:

	Yes	**No**
Information sheet given	☐	☐
Study consent form signed x3	☐	☐

Admission Date and time: GP or A+E
Date of onset of symptoms:
Breathlessness: Y / N Wheeze: Y/ N
Cough: Y/ N Chest Tightness: Y / N
Sputum: Y / N Colour: Chest Pain: Y / N
Obvious trigger: Y / N Give details:
Night-time wakening: Y / N Frequency of reliever use:

Intercurrent menstruation Y / N if yes give dates:
PMHx and FHx:

Smoking Hx:

Asthma Hx including admissions and exacerbations last 2 years:

GINA classification of asthma severity by clinical features before treatment (worst feature determines severity)	
Intermittent	
Symptoms < once a week	
Brief exacerbations	
Nocturnal symptoms not > twice a month	
FEV_1 or PEFR ≥ 80% predicted	
PEFR or FEV_1 variability <20%	
Mild Persistent	
Symptoms > once a week but < once a day	
Exacerbations may affect activity and sleep	
Nocturnal symptoms > twice a month	
FEV_1 or PEFR ≥ 80% predicted	
PEFR or FEV_1 variability <20-30%	
Moderate Persistent	
Symptoms daily	
Exacerbations may affect activity and sleep	
Nocturnal symptoms more than once a week	
Daily use of inhaled SABA	
FEV_1 or PEFR 60-80% predicted	
PEFR or FEV_1 variability >30%	
Severe Persistent	
Symptoms daily	
Frequent exacerbations	
Frequent nocturnal asthma symptoms	
Limitation of physical activities	
FEV_1 or PEFR ≤ 60% predicted	
PEFR or FEV_1 variability > 30 %	

Signature:_____ Date:___/___/_____ 2

Addressograph

Name & Hospital No.

Address

D.O.B.

Subject's study number:

Usual Medications

Drug	Dose	Frequency

Acute medications

Drug	Dose	Frequency	Date/time 1st dose

PEFR:_____ Pred PEFR:_____ Best PEFR:_____
O_2 sats: FiO_2:
BP: Pulse:
Height: Weight:
CXR normal Y / N Who?_____

Examination findings:

Signature:_____ Date:___/___/_____

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma? – Part 1 – Observational Study
Case Record Form_admission_version 1.3_01/03/2011 REC no: 10/H0505/59

Addressograph

Name & Hospital No.

Address

D.O.B.

Subject's study number:

Admission blood results:				Date and time taken:			
Hb		INR		Bili		Urea	
WCC		APTR		ALP		Creat	
Plats		D-dimer*		AST			
MCV		Fibrinogen		Alb			
Neut		IgE		$adjCa^{2+}$		Urine Creat	
Eosin		CRP		Na^+			
Mono		Gluc		K^+			

Blood tests recorded by: (sign and date)_____
* D-dimer needs special request

Date and time of spirometry:

	Predicted	Actual	% predicted
FEV_1			
FVC			
FEV_1/FVC			

Date and time of urine collection: GP letter sent_____☐

Date and time of sputum collection:

Expectorated sputum: Y / N

Blood tests done including study bloods: Y / N

Exhaled nitric oxide performed: Y / N Result:_____
By whom? (date, time and sign)

Clexane given before sample collection: Y / N If so what time:_____

Wards admitted to (list in order):

Ward assessed and samples taken:

Date of discharge:

Duration of admission:

Signature:_____ Date:___/___/_____

Case Record Form – Follow up

Addressograph
Name & Hospital No.
Address
D.O.B.

Subject's study number:

Date Seen: ___/___/_____

Wheeze:
Breathlessness:
Chest tightness:
Cough:
Sputum:
Chest Pain:
Night-time wakening:
Reliever use:
Returned to normal?

Intercurrent menstruation Y / N if yes give dates:

Current medications:		
Drug	**Dose**	**Frequency**

Acute medications continued or date stopped			
Drug	**Dose**	**Frequency**	**Date stopped/ongoing**

Total duration oral steroids:
Total duration antibiotics:

Signature:_____ Date:___/___/_____

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma? – Part 1 – Observational Study
Case Record Form_follow up_version 1.2_28/10/2010 REC no: 10/H0505/59

Addressograph
Name & Hospital No.
Address
D.O.B.

Subject's study number:

PEFR:
O_2 sats:
FiO_2:
BP:
Pulse:

Examination findings:

Date and time of spirometry:

	Predicted	**Actual**	**% predicted**
FEV_1			
FVC			
FEV_1/FVC			

PEFR diary reviewed: Y / N Who?_____

PEFR stable: Y / N

PEFR back to best or predicted: Y / N

Skin prick testing: Date .....

Test substance	Result (mm)		
Histamine	.....	Tree pollen	.....
Cat	.....	House dust mite	.....
Dog	.....	*Aspergillus*	.....
Grass pollen	.....	Control	.....

Signature:_____ Date:___/___/_____

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma? – Part 1 – Observational Study
Case Record Form_follow up_version 1.2_28/10/2010 REC no: 10/H0505/59

Addressograph

Name & Hospital No.

Address

D.O.B.

Subject's study number:

Date and time of urine collection:

Date and time of sputum collection:

Blood tests done including study bloods: Y / N

Exhaled nitric oxide performed: Y / N Result:_____
By whom? (date, time and sign)

Expectorated or induced sputum

If induced sputum: - Hospital consent signed Y / N
- Study consent signed Y / N

Follow Up blood results:				Date and time taken:			
Hb		INR		Bili		Urea	
WCC		APTR		ALP		Creat	
Plats		D-dimer*		AST			
MCV		Fibrinogen		Alb			
Neut		IgE		adjCa^{2+}		Urine Creat	
Eosin		CRP		Na^{+}			
Mono		Gluc		K^{+}			

Blood results transcribed by:_____Date:_____

If smoking Hx between 10 and 20 pack years – Date/time of PFT:			
	Predicted	Actual	%
TLC			
RV			
VC			
TLco			
VA			
Kco			

Signature:_____ Date:___/___/_____

Case Record Form – Visit 1

Addressograph
Name & Hospital No.
Address
D.O.B.

Subject's study number:

Date Seen: ___/___/_____

Tel No:

Next of Kin: N.O.K. Tel No:

Inclusion Criteria:	**Yes**	**No**
• Moderate asthma >1yr (500-1000mcg BDP)_____	☐	☐
• Severe asthma >1yr (1000-2000mcg BDP +/- oral steroids)____	☐	☐
• Age 18-70 years_____	☐	☐

Exclusion Criteria:	**Yes**	**No**
• Current or ex-smokers with >20 pack year history_____	☐	☐
• Significant cardiopulmonary co-morbidities_____	☐	☐
• Clinically significant neurological, renal, endocrine, gastrointestinal, hepatic or haematological abnormalities uncontrolled with standard treatment.	☐	☐
• Regular use of an anticoagulant_____	☐	☐
• Bleeding diatheses_____	☐	☐
• Alcohol and recreational drug abuse_____	☐	☐
• Treatment with other immunomodulators_____	☐	☐
• Concurrent participation in another interventional study_____	☐	☐
• Abnormal chest xray_____	☐	☐
• Asthma exacerbation in the last 4 weeks_____	☐	☐

Signature:_____ Date:___/___/_____ 1

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma? – Part 2 – Longitudinal Study
Case Record Form_initial visit_version 1.4_01/03/2011 REC no: 10/H0505/59

Addressograph
Name & Hospital No.
Address
D.O.B.

Subject's study number:

	Yes	**No**
Information sheet given_____	☐	☐
Study consent form signed x3_____	☐	☐

Breathlessness: Y / N
Cough: Y/ N
Sputum: Y / N Colour:
Night-time wakening: Y / N

Wheeze: Y/ N
Chest Tightness: Y / N
Chest Pain: Y / N
Frequency of reliever use:

Intercurrent menstruation Y / N if yes give dates:

PMHx and FHx:

Smoking Hx:

Asthma Hx including admissions and exacerbations last 2 years:

Triggers:

GINA classification of asthma severity by clinical features before treatment (worst feature determines severity)	
Intermittent	
Symptoms < once a week	
Brief exacerbations	
Nocturnal symptoms not > twice a month	
FEV_1 or PEFR ≥ 80% predicted	
PEFR or FEV_1 variability <20%	
Mild Persistent	
Symptoms > once a week but < once a day	
Exacerbations may affect activity and sleep	
Nocturnal symptoms > twice a month	
FEV_1 or PEFR ≥ 80% predicted	
PEFR or FEV_1 variability <20-30%	
Moderate Persistent	
Symptoms daily	
Exacerbations may affect activity and sleep	
Nocturnal symptoms more than once a week	
Daily use of inhaled SABA	
FEV_1 or PEFR 60-80% predicted	
PEFR or FEV_1 variability >30%	
Severe Persistent	
Symptoms daily	
Frequent exacerbations	
Frequent nocturnal asthma symptoms	
Limitation of physical activities	
FEV_1 or PEFR ≤ 60% predicted	
PEFR or FEV_1 variability > 30 %	

Signature:_____ Date:___/___/_____ 2

Addressograph

Name & Hospital No.

Address

D.O.B.

Subject's study number:

Usual Medications

Drug	Dose	Frequency

PEFR:_____ Pred PEFR:_____ Best PEFR:_____
O_2 sats:
FiO_2:
BP:
Pulse:
Height:
Weight:
CXR normal Y / N Who?_____
Examination findings:

Date and time of spirometry:

	Predicted	Actual	% predicted
FEV_1			
FVC			
FEV_1/FVC			

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma? – Part 2 – Longitudinal Study
Case Record Form_initial visit_version 1.4_01/03/2011 REC no: 10/H0505/59

Addressograph

Name & Hospital No.

Address

D.O.B.

Subject's study number:

GP letter sent_____☐

Blood tests taken:Y / N (D-dimer needs special request) Time & date of bloods:

PEFR meter and diary given:Y / N

Urine sample taken: Y / N Time & date of urine:

Sputum sample taken: Y / N Time & date of sputum:

Induced / expectorated sputum?

Exhaled nitric oxide performed: Y / N Result:_____
By whom? (date, time and sign)

Skin prick testing: Date .....

Test substance	Result (mm)		
Histamine	.....	Tree pollen	.....
Cat	.....	House dust mite	.....
Dog	.....	*Aspergillus*	.....
Grass pollen	.....	Control	.....

If smoking Hx between 10 and 20 pack years – Date/time of PFT:

	Predicted	Actual	%
TLC			
RV			
VC			
TLco			
VA			
Kco			

Addressograph

Name & Hospital No.

Address

D.O.B.

Case Record Form – Visit 2 Exacerbation

Subject's study number:

Date Seen: ___/___/_____

Onset of symptoms:

Wheeze: Y / N
Breathlessness: Y / N
Chest tightness: Y / N
Cough: Y / N
Sputum: Y / N Colour:
Chest Pain: Y / N
Night-time wakening: Y / N
Reliever use:
Intercurrent menstruation Y / N if yes give dates:

Feels like an exacerbation to patient? Y / N

Seen GP? Y / N

PEFR:
O_2 sats:
FiO_2:
BP:
Pulse:

PEFR diary reviewed: Y / N Who?_____

PEFR stable: Y / N

Acute medications started/doses changed

Drug	Dose	Frequency	Date started

Signature:_____ Date:___/___/_____

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma? – Part 2 – Longitudinal Study
Case Record Form_exacerbation_version 1.2_01/03/2011 REC no: 10/H0505/59

Addressograph

Name & Hospital No.

Address

D.O.B.

Subject's study number:

Date and time of spirometry:

	Predicted	Actual	% predicted
FEV_1			
FVC			
FEV_1/FVC			

Date and time of sputum collection:

Expectorated sputum: Y / N

Urine samples collected: Y / N Date & time of urine:

Drugs given at this consultation

Drug	Dose	Frequency	Planned duration

Urine pots given: Y / N

Blood tests taken: Y / N (D-dimer needs special request)

Blood tests done including study bloods: Y / N

Exhaled nitric oxide performed: Y / N Result:_____
By whom? (date, time and sign)

Date and time for next visit:_____

Signature:_____ Date:___/___/_____

Addressograph

Name & Hospital No.

Address

D.O.B.

Subject's study number:

Baseline blood results from visit 1: Date and time taken:							
Hb		INR		Bili		Urea	
WCC		APTR		ALP		Creat	
Plats		D-dimer*		AST			
MCV		Fibrinogen		Alb			
Neut		IgE		adjCa^{2+}		Urine Creat	
Eosin		CRP		Na^{+}			
Mono		Gluc		K^{+}			

Blood results transcribed by:_____Date:_____

Signature:_____ Date:___/___/_____

Addressograph

Name & Hospital No.

Address

D.O.B.

Case Record Form – Visit 3 Recovery

Subject's study number:

Date Seen: ___/___/_____

Recovery of symptoms by which date:

Wheeze: Y / N
Breathlessness: Y / N
Chest tightness: Y / N
Cough: Y / N
Sputum: Y / N Colour:
Chest Pain: Y / N
Night-time wakening: Y / N
Reliever use:

Feels fully recovered: Y / N

Intercurrent menstruation Y / N if yes give dates:

PEFR:
O_2 sats:
FiO_2:
BP:
Pulse:

PEFR diary reviewed: Y / N Who?_____

PEFR stable: Y / N

Acute medications stopped/doses adjusted			
Drug	**Dose**	**Frequency**	**Date stopped**

Are medications back to normal? Y / N if not give details in table above.

Signature:_____ Date:___/___/_____

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma? – Part 2 – Longitudinal Study
Case Record Form_recovery_version 1.2_01/03/2011 REC no: 10/H0505/59

Addressograph

Name & Hospital No.

Address

D.O.B.

Subject's study number:

Examination findings:

Date and time of spirometry:

	Predicted	**Actual**	**% predicted**
FEV_1			
FVC			
FEV_1/FVC			

Date and time of sputum collection:

Expectorated or induced sputum:

Exhaled nitric oxide performed: Y / N Result:_____
By whom? (date, time and sign)

Urine samples collected: Y / N Date and time of urine:

Blood tests taken: Y / N (* D-dimer needs special request)

Exacerbation blood results:				**Date and time taken:**			
Hb		INR		Bili		Urea	
WCC		APTR		ALP		Creat	
Plats		D-dimer*		AST			
MCV		Fibrinogen		Alb			
Neut		IgE		$adjCa^{2+}$		Urine Creat	
Eosin		CRP		Na^+			
Mono		Gluc		K^+			

Blood results transcribed by:_____Date:_____

Signature:_____ Date:___/___/_____

Addressograph
Name & Hospital No.
Address
D.O.B.

Subject's study number: [ ]

Recovery blood results:				**Date and time taken:**			
Hb		INR		Bili		Urea	
WCC		APTR		ALP		Creat	
Plats		D-dimer*		AST			
MCV		Fibrinogen		Alb			
Neut		IgE		adjCa^{2+}		Urine Creat	
Eosin		CRP		Na^{+}			
Mono		Gluc		K^{+}			

Blood results transcribed by:_____Date:_____

Signature:_____ Date:___/___/_____

Induced Sputum Test Information Sheet

Sputum Induction is a procedure that requires your active participation. The success of collecting an adequate sample from the airways is solely dependant on your ability to vigorously and deeply cough to produce a sample. This is a 20 minute procedure (maximum) during which you must wear a nose clip and breath in a strong salty mist from the nebuliser.

Prior to commencing the procedure:

- **We will test your lung function by asking you to blow into the spirometer and the peak flow meter. We will record these values**
- **We will then give you Salbutamol (Ventolin) via a spacer device, 2 puffs (200mcgs)**
- **After 10 minutes we will repeat your lung function and use this as our baseline figure throughout the rest of the procedure**

During the procedure:

- **We will ask you to sit and breath in a salty mist from the nebuliser wearing a nose clip**
- **Every 5 minutes whilst being encouraged by the technician/nurses, you will come off the mouthpiece to clear saliva from your mouth by spitting into the bowl supplied**
- **We will then ask you to rinse your mouth with the water supplied before coughing vigorously into the Petri dish**
- *If an adequate sample is obtained the procedure will be stopped otherwise it will be continued at 5 minute intervals for up to 20 minutes*
- **After every 5 minutes of nebulisation we will ask you to spit, rinse and cough. We will then ask you to blow into the spirometer and the peak flow meter to monitor for any changes in your lung function**
- ***If there is a significant drop in lung function we will stop the procedure***
- **You can clear your mouth of saliva at any time**

You can cough into the Petri dish at any time if you feel the need to cough

Symptoms you may experience may include:

- **A salt water after taste**
- **Coughing**
- **A sense of needing to swallow**
- **Throat irritation**

And more rarely:

- **Shortness of breath**
- **Wheeze**
- **Chest tightness**
- **Light-headedness**
- **Nausea**
- **Headache**

If the investigator thinks your airways may constrict because of the induction we may use a modified protocol using a lower concentration on salt.

Please inform the technician/nurse immediately if you should become symptomatic

Respiratory Centre
Portsmouth Hospitals NHS
NHS Trust

Prof A J Chauhan FRCP PhD
Consultant Respiratory Physician
Dr J Owen MRCP
Clinical Research Fellow
Mrs Su Kerley
Respiratory Research Nurse
Reception: 023 9228 6665
Secretary: 023 9228 6000 ext 1386
Nurse: 023 9228 6000 ext 4108

Respiratory Centre
Queen Alexandra Hospital
Cosham PO6 3LY
Main Switchboard:023 9228 6000

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?

Induced Sputum Consent Form

Name	D.O.B.	Study Number	Date

	Please initial
I have read and understand the induced sputum test information sheet including the potential risks involved with the test	

I agree to taking part in the induced sputum test	

Name of Patient	Date	Signature

Investigator	Date	Signature

Addressograph	Study No:
Name & Hospital No.	
Address	Date:
D.O.B.	Initial Visit / Recovery

- **SPUTUM INDUCTION**

SPUTUM INDUCTION/PROCEDURAL WORKSHEET
Standard

Nurse/Tech:	Physician:

	Time	PEFR	FEV1
Baseline			
Post Salbutamol			
20% fall (record value)	*Please make sure the technique is good, if in doubt, repeat*		

Strength of Hypertonic Saline used		Expiry Date	

Minutes	PEFR	FEV1
5		
10		
15		
20		

Please indicate (√) reason (s) for discontinuing induction

Adequate sample obtained		Chest tightness/breathlessness	
Significant fall in FEV1/PEFR		Excessive coughing	
Other		Please record reason:	

Comments:

Are markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?
Induced sputum protocol_version 1.2_28/07/2010 REC no: 10/H0505/59

Addressograph	Study No:
Name & Hospital No.	
Address	Date:
D.O.B.	Initial Visit / Recovery

SPUTUM INDUCTION/PROCEDURAL WORKSHEET
For subjects who are *at risk of bronchoconstriction*

Nurse/Tech:	Physician:

	Time		FEV1
Baseline			
Post Salbutamol			
20% fall (record value)	*Please make sure the technique is good at each time point, if in doubt, repeat*		

0.9% NaCl Normal Saline √ Expiry Date

Minutes	FEV1	% fall from baseline
30 sec		
1 min		
5 min		

3% Saline √ Expiry Date

Minutes	FEV1	% fall from baseline
30 sec		
1 min		
2 min		

4.5% Saline √ Expiry Date

Minutes	FEV1	% fall from baseline
30 sec		
1 min		
2 min		
4 min		
8min		

Continued Over...

Addressograph
Name & Hospital No.
Address
D.O.B.

Study No:

Date:

Initial Visit / Recovery

Please indicate (√) reason (s) for discontinuing induction			
Adequate sample obtained		**Chest tightness/breathlessness**	
Significant fall in FEV1/PEFR		**Excessive coughing**	
Other		**Please record reason:**	
Comments:			

FEV1 at discharge from Unit ☐ . ☐☐

Actual Time (24hr) ☐☐ : ☐☐

Investigator signature & date:

Appendix 19 – Peak flow and symptom diary

Are Markers of fibrin turnover in blood and urine increased before and during exacerbations of asthma?
Peak flow and symptom record (version 1.1 11/5/2010) **REC no: 10/H0505/59**

Name:		Hospital number:	
D.O.B:		Study Number:	

Date														
	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm
700														
650														
600														
550														
500														
450														
400														
350														
300														
250														
200														
150														
100														
50														
0														
Wheeze (Y/N)														
Night waking (Y/N)														
Reliever use (No. of puffs/day)														
Chest tightness (Y/N)														
Breathlessness (Y/N)														
Oral steroid dose (if taking)														
Urine sample (please tick)														
Menstruating (please tick)														

Appendix 20 – Blood and urine tests by PHT laboratory

Blood and urine tests performed by PHT laboratory			
Parameter	**Reference range**	**Analyser**	**Supplier**
Haematology			
Full blood count (FBC)		Beckman Coulter LH 750	Beckman Coulter (UK) Ltd, Oakley Court, Kingsmead Business Park, London Road, High Wycombe, HP11 1JU, UK
Haemoglobin (Hb)	12.0-16.0 g/dl		
White cell count (WCC)	4.0-11.0 $x10^9$/l		
Platelets (Plats)	150-400 $x10^9$/l		
Mean cell volume (MCV)	80.0-99.0 fl		
Neutrophils (Neut)	2.0-7.5 $x10^9$/l		
Eosinophils (Eosin)	0.0-0.4 $x10^9$/l		
Monocytes (Mono)	0.2-0.8 $x10^9$/l		
Clotting			
International normalised ratio (INR)	0.8-1.2	IL ACL Top	Diamond Diagnostics, 333 Fiske Street, Holliston, MA 01746, USA
Activated partial thromboplastin ratio (APTR)	0.8-1.2		
D-dimer	0-0.5 µgFEU/ml		
Fibrinogen	1.5-3.5 g/l		
Immunology			
Immunoglobulin E (IgE)	0-81 IU/ml	Phadia 250	Phadia AB, P.O. Box 6460, SE-751 37 UPPSALA, Sweden
Biochemistry			
C-reactive protein (CRP)	0-5 mg/l	DxC 800 Synchron	Beckman Coulter (UK) Ltd, Oakley Court, Kingsmead Business Park, London Road, High Wycombe, HP11 1JU, UK
Serum glucose (Gluc)	3.5-6.0 mmol/l		
Random urine creatinine (Urine creat)	mmol/l		
Liver function tests (LFT)			
Bilirubin (Bili)	3-20 µmol/l		
Alkaline phosphatase (ALP)	30-130 iu/l		
Aspartate transaminase (AST)	12-40 iu/l		
Bone profile			
Albumin (Alb)	35-48 g/l		
Adjusted calcium ($adjCa^{2+}$)	2.15-2.60 mmol/l		
Urea and electrolytes (U+E)			
Sodium (Na^+)	135-146 mmol/l		
Potassium (K^+)	3.5-5.0 mmol/l		
Urea	2.9-7.1 mmol/l		
Creatinine (Creat)	40-90 µmol/l		

Appendix 21 – Blood and urine tests by BNHFT laboratory

Blood and urine tests performed by BNHFT laboratory			
Parameter	**Reference range**	**Analyser**	**Supplier**
Haematology			
Full blood count (FBC)		Siemens Advia 2120	Siemens Healthcare Diagnostics Inc. 511 Benedict Avenue/Tarrytown, NY 10591, USA
Haemoglobin (Hb)	110-165 g/l		
White cell count (WCC)	4.0-11.0 $x10^9$/l		
Platelets (Plats)	150-500 $x10^9$/l		
Mean cell volume (MCV)	79-98 fl		
Neutrophils (Neut)	1.5-8 $x10^9$/l		
Eosinophils (Eosin)	0-0.8 $x10^9$/l		
Monocytes (Mono)	0.2-0.8 $x10^9$/l		
Clotting			
International normalised ratio (INR)	0.8-1.2	Diagnostica Stago – Destiny Max	Diagnostica Stago UK Ltd, 2 Theale Lakes Business Park, Moulden Way, Theale, RG7 4GB, UK
Activated partial thromboplastin time (APTT)	25.4-34.6s		
D-dimer	0-0.5 mg/l		
Fibrinogen	1.5-4 g/l		
Immunology			
Immunoglobulin E (IgE)	Tested at PHT – see above		
Biochemistry			
C-reactive protein (CRP)	<2 mg/l	Siemens Advia 2400	Siemens Healthcare Diagsnostics Inc. 511 Benedict Avenue/Tarrytown, NY 10591, USA
Serum glucose (Gluc)	3.8-6 mmol/l		
Random urine creatinine (Urine creat)	mmol/l		
Liver function tests (LFT)			
Bilirubin (Bili)	0-17 µmol/l		
Alkaline phosphatase (ALP)	35-110 iu/l		
Alanine transaminase (ALT)	0-60 iu/l		
Bone profile			
Albumin (Alb)	32-48 g/l		
Adjusted calcium (adjCa^{2+})	2.12-2.62 mmol/l		
Urea and electrolytes (U+E)			
Sodium (Na^+)	134-147 mmol/l		
Potassium (K^+)	3.5-5 mmol/l		
Urea	2.6-6 mmol/l		
Creatinine (Creat)	62-124 µmol/l		

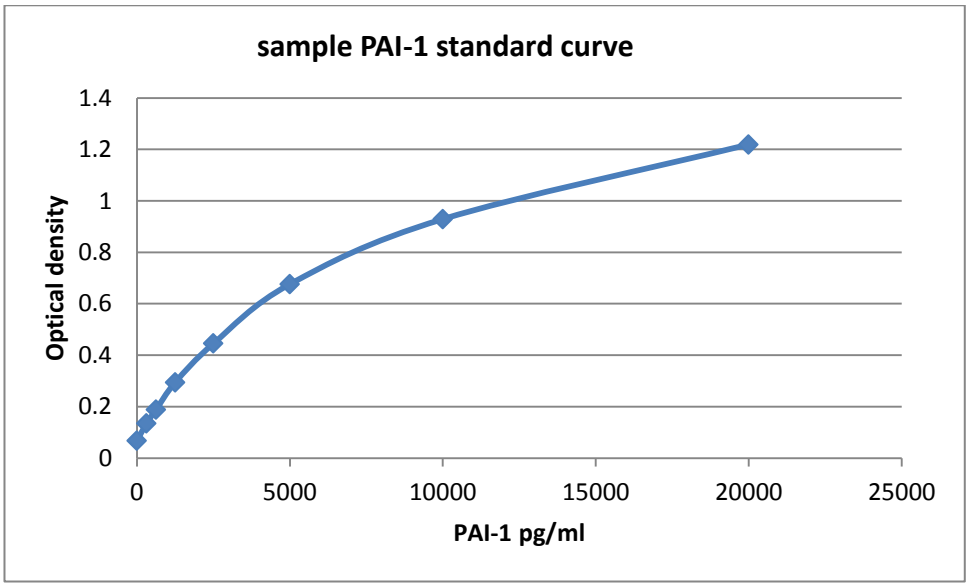
Appendix 22 – TMB substrate solution for plasma ELISA

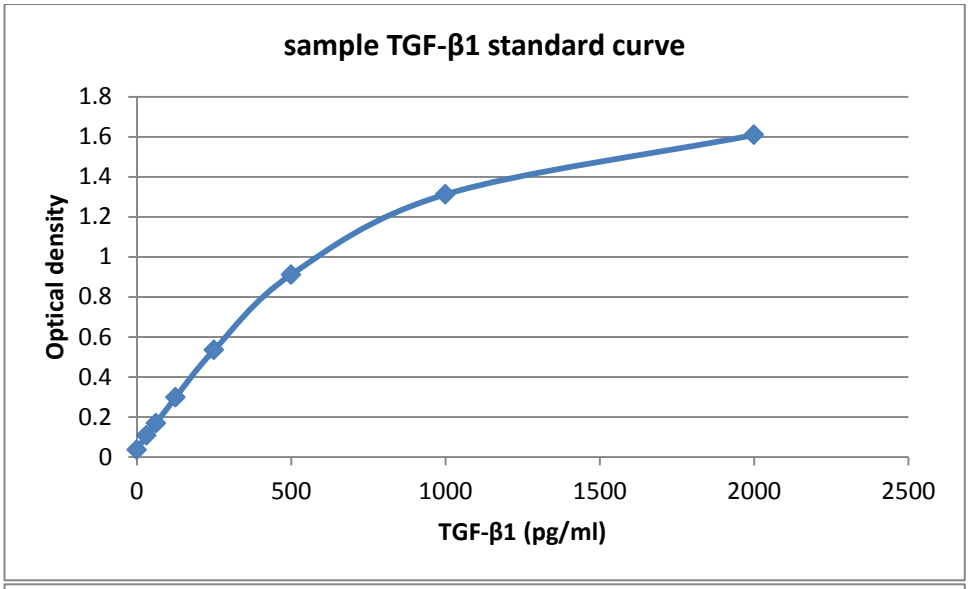
Substrate buffer = 1.5g sodium acetate dissolved in 80ml de-ionised H_2O, adjusted to pH 5.5 with glacial acetic acid. Made up to 100ml with de-ionised H_2O then refrigerate for up to 2 weeks at $4^{o}c$

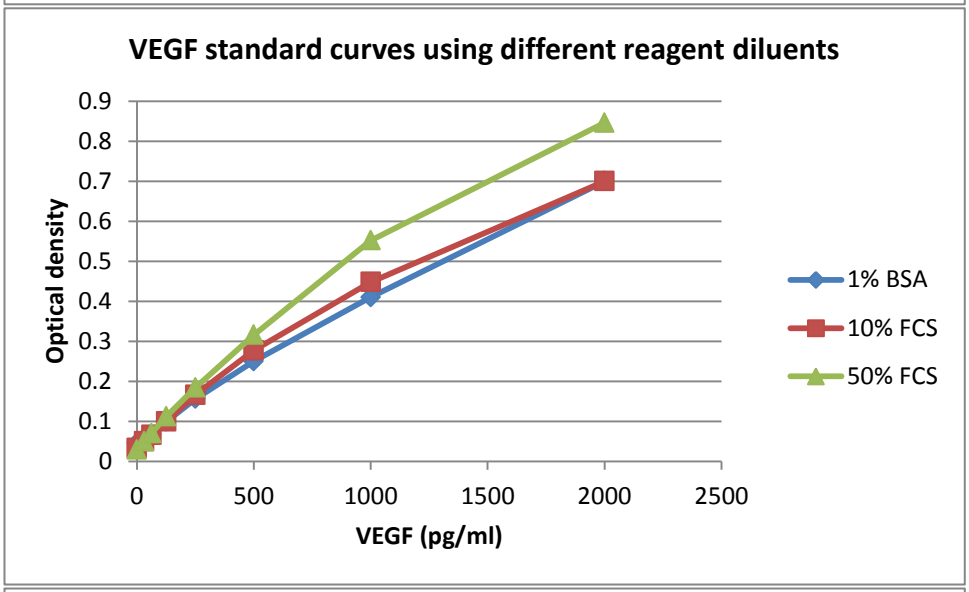
Tetramethylbenzidine (TMB) stock solution = 30mg TMB dissolved in 5ml DMSO, store up to 1 month at room temperature protected from sunlight

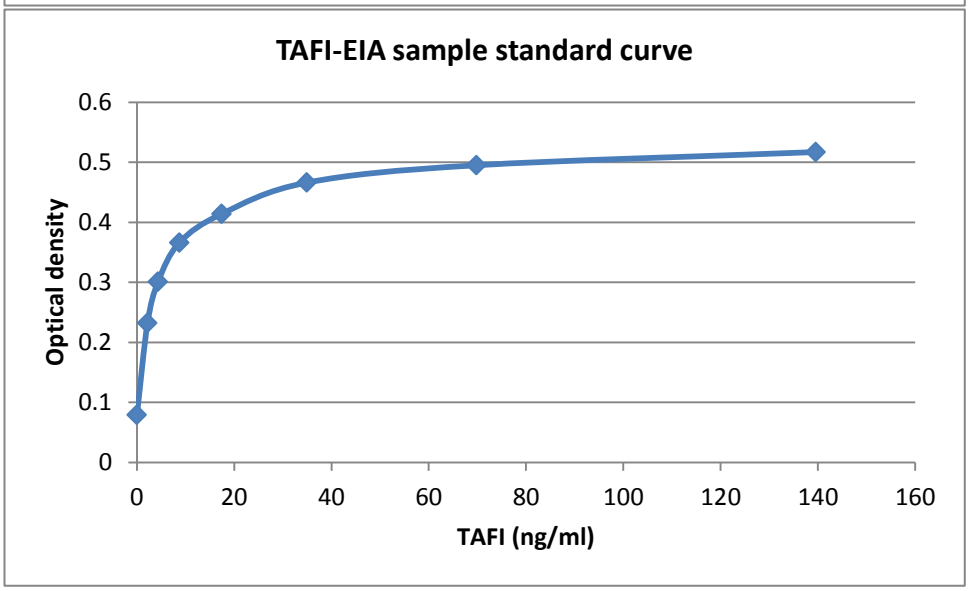
Substrate solution (for each 96 well plate) = 12ml substrate buffer + 200µl TMB stock solution + 1.2µl H_2O_2 30% solution

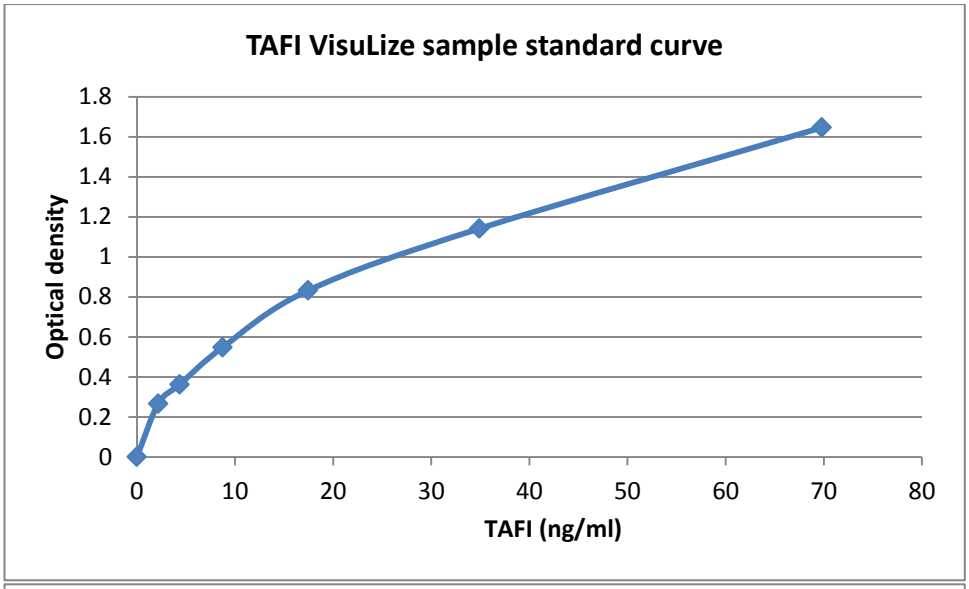
Appendix 23 – Sample standard curves

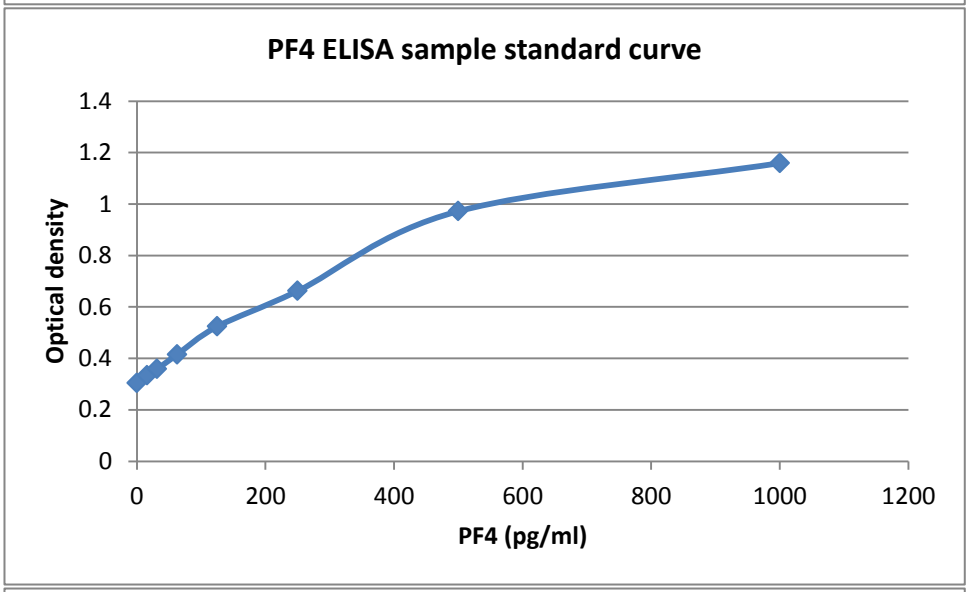


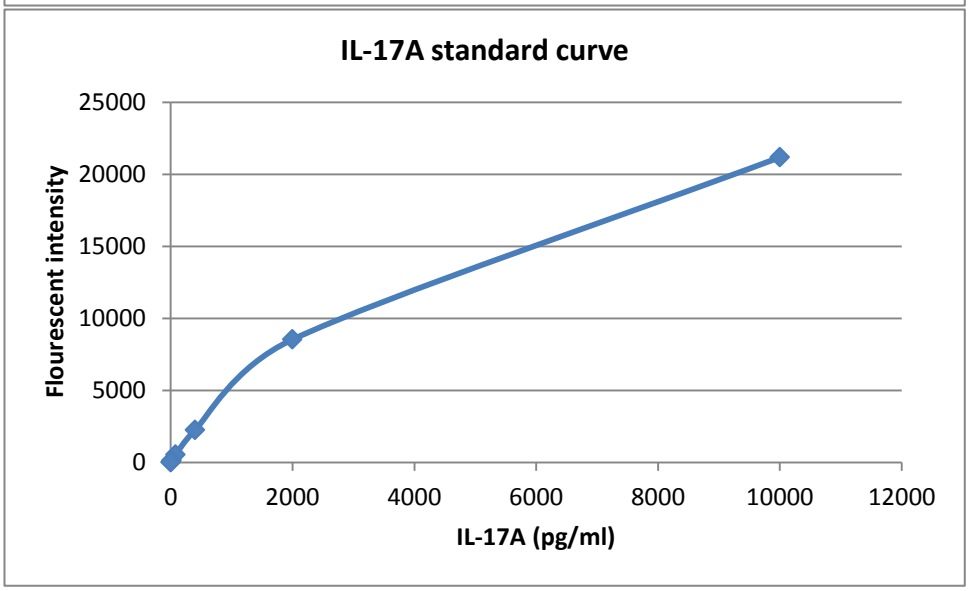

sample TGF-β1 standard curve
Optical density
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
500
1000
1500
2000
2500
TGF-β1 (pg/ml)


VEGF standard curves using different reagent diluents
Optical density
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
500
1000
1500
2000
2500
VEGF (pg/ml)
1% BSA
10% FCS
50% FCS


TAFI-EIA sample standard curve
Optical density
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
TAFI (ng/ml)


TAFI VisuLize sample standard curve
Optical density
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
TAFI (ng/ml)


PF4 ELISA sample standard curve
Optical density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
1000
1200
PF4 (pg/ml)


IL-17A standard curve
Flourescent intensity
25000
20000
15000
10000
5000
0
0
2000
4000
6000
8000
10000
12000
IL-17A (pg/ml)

Appendix 24 – UPR16

FORM UPR16

Research Ethics Review Checklist

University of Portsmouth

Please complete and return the form to Research Section, Quality Management Division, Academic Registry, University House, with your thesis, prior to examination

Postgraduate Research Student (PGRS) Information			**Student ID:**	602626 01
Candidate Name:	Dr Jonathan Owen			
Department:	Pharmacy & biomedical Sciences	**First Supervisor:**	Prof Jan Shute	
Start Date: (or progression date for Prof Doc students)	Feb 2010			

Study Mode and Route:	Part-time ☑ Full-time ☐	MPhil ☐ MD ☑ PhD ☐	Integrated Doctorate (NewRoute) ☐ Prof Doc (PD) ☐

Title of Thesis:	Are markers of fibrin turnover in blood and urine increased before and during exacerbation of asthma?
Thesis Word Count: (excluding ancillary data)	32,362

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

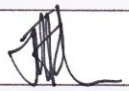
Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)

a) **Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?**	YES/~~NO*~~
b) **Have all contributions to knowledge been acknowledged?**	YES/~~NO*~~
c) **Have you complied with all agreements relating to intellectual property, publication and authorship?**	YES/~~NO*~~
d) **Has your research data been retained in a secure and accessible form and will it remain so for the required duration?**	YES/~~NO*~~
e) **Does your research comply with all legal, ethical, and contractual requirements?**	YES/~~NO*~~

*Delete as appropriate

UPR 16 (2013) – November 2013

Candidate Statement:	
I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)	
Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	10/H0505/59
Signed: *(Student)* 	**Date:** 18/09/2014
If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain why this is so:	
Signed: *(Student)*	**Date:**